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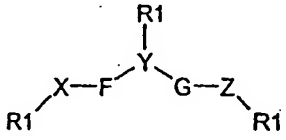

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(57) Abstract: Use of chemical compounds or selections of chemical compounds (libraries) of the general formula (I): for *in vivo* methods for testing or validating the physiological importance and/or the therapeutic or pharmacological potential of biological target molecules, notably proteins such as, e.g., receptors and especially 7TM receptors in test animals expressing the biological target molecule with, notably, a silent, engineered metal-ion site. Use of specific metal-ion binding sites of a generic nature in specific biological target molecules such as, e.g. transmembrane proteins wherein the metal-ion binding site is capable of forming a complex with a metal ion is also described. Chemical compounds or libraries suitable for use in methods for improving the *in vivo* pharmacokinetic behaviour of metal-ion chelates (e.g. the absorption pattern, the plasma half-life, the distribution, the metabolism and/or the elimination of the metal-ion chelates). In order to improve the efficacy of the metal-ion chelates impact on the biological target molecule after administration of the metal-ion chelate *in vivo* to a test animal it is advantageous e.g. to increase the time period during which the metal-ion chelate is in the circulatory system and/or localised at the target. Metal-ion chelating compounds, which are designed to be suitable for use in a target validation process according to the invention and to libraries of at least two or more of such metal-ion chelating compounds are disclosed.

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CHEMICAL LIBRARIES USEFUL FOR DRUG DISCOVERY PROCESSES

Field of the invention

The present invention relates to chemical libraries of chemical compounds containing at least two heteroatoms such as, e.g. N, O, S, Se and/or P. The chemical compounds are capable of forming complexes with a metal ion, i.e. in one aspect the invention relates to a library of chelators. In those cases where the chemical compounds are present in the form of metal-ion complexes, the invention relates to a library of chelates. The libraries are useful in a drug discovery method disclosed in PCT/EP00/13389 which relates to a novel method useful for identifying small organic molecule ligands (in the following also denoted "compounds") for binding to specific sites on biological target molecules such as proteins, nucleic acids, carbohydrates, nucleoproteins, glycoproteins and glycolipids. The compounds are capable of interacting with the biological target molecule, in particular with a protein, in such a way as to modify the biological activity thereof.

The invention further relates to libraries that are suitable for use in methods of identifying compounds acting as receptor-ligands of biological target molecules such as, e.g., proteins, the method comprises the introduction of metal ion binding sites into the biological target molecules: The method includes identifying compounds that bind to orphan receptors.

Small organic receptor-ligands identified according to the methods of the present invention find use, for example, as novel therapeutic drug compounds or drug lead compounds, enzyme inhibitors, labelling compounds, diagnostic reagents, affinity reagents e.g. for protein purification etc.

Introduction

The initial phase in developing novel biologically active compounds such as, e.g., therapeutically or propylactically active drug compounds is to identify and characterize one or more binding receptor-ligand(s) for a given biological target. Many molecular techniques have been developed and are currently being employed for identifying novel receptor-ligands or compounds that bind to the biological target. In the following proteins are used as an example on a biological target molecule.

Proteins as drug targets

Most drug compounds act by binding to and altering the function of proteins. These can be intracellular proteins such as, for example enzymes and transcription factors, or they can be extracellular proteins, for example enzymes, or they can be membrane proteins. Membrane proteins constitute a numerous and varied group whose function is either structural, for example being involved in cell adhesion processes, or the membrane proteins are involved in intercellular communication and communication between the cell exterior and the interior by transducing chemical signals across cell membranes, or they facilitate or mediate transport of compounds across the lipid membrane. Membrane proteins are for instance receptors and ion channels to which specific chemical messengers termed receptor-ligands bind resulting in the generation of a signal, which gives rise to a specific intracellular response (this process is known as signal transduction). Membrane proteins can, for example, also be enzymes that are associated to the membrane for functional purposes, e.g. proximity to their substrates. Most membrane proteins are anchored in the cell membrane by a sequence of amino acid residues, which are predominantly hydrophobic to form hydrophobic interactions with the lipid bilayer of the cell membrane. Such membrane proteins are also known as integral membrane proteins. In most cases, the integral membrane proteins extend through the cell membrane into the interior of the cell, thus comprising an extracellular domain, one or more transmembrane domains and an intracellular domain.

A large fraction of current drugs act on membrane proteins and among these the majority are targeted towards the G protein coupled receptors (GPCR) with their seven transmembrane segments, also called 7TM receptors.

Identification of lead compounds in drug discovery

Drug discovery traditionally involves a process where a lead compound first is identified and then subsequently chemically optimised for high affinity and selectivity for the protein target (or another biological target molecule) and optimised for other drug-like properties such as lack of toxic effects and desirable pharmacokinetics.

Recent drug development has focused on screening of large libraries of chemical compounds in order to identify lead compounds, which are capable of either up-regulating (called agonists) or down-regulating the activity of the protein target (called antagonists), as required. Screening has usually been performed in a "shot-gun" fashion by setting up

an assay for screening large numbers of compounds, e.g. large files of compounds or compounds in combinatorial libraries, in order to identify compounds with the desired activity. The subsequent chemical optimization of the lead compounds obtained from such screening procedures has been performed very much in a trial-and-error fashion and has been quite cumbersome and resource-demanding, involving procedures such as described by E. Sun and F.E. Cohen, *Gene* 1993 137(1), 127-32, or J. Kuhlmann, *Int J Clin Pharmacol Ther.* 1999 37(12), 575-83. A major disadvantage of the drug discovery process is that it is difficult to identify active compounds with sufficient selectivity and specificity for a given target protein or in many cases it is even difficult at all to identify suitable lead compounds, for example for interfering with protein-protein interactions.

Optimization of lead compounds to high affinity receptor-ligands

Through the generation of chemical analogues of the lead compound and testing of these for binding or activity on the biological target molecules such as a protein target, the lead compound is gradually improved in affinity for the target. Also this process in to a large degree done by trial-and-error, although the medicinal chemist usually is guided by a gradually increasing knowledge in the structure activity relationship (SAR) of the compounds, i.e. the observation of which modification at which site in the compound that increase or decrease the activity of the compound. The SAR can provide a great deal of information regarding the nature of receptor-ligand-receptor interactions, but no detailed information about the location and actual chemical nature of the binding site in the target protein is provided. A number of closely related chemical structures are used to direct the orientation of the receptor-ligand within the putative binding cavity and to determine what part of the receptor-ligand is involved in binding to the receptor. This technique has its limitations due to the fact that changing the structure of the receptor-ligand may result in a actual change in the binding site of the receptor (Mattos et al. *Struct. Biol.*, 1995 1:55-58), a fact which obviously still would be un-know to the medicinal chemist. Thus, in most cases the lack of knowledge of the precise molecular interaction with the receptor of the lead compounds found by chemical screening has prevented a rational chemical approach to the optimisation of the lead compound.

Identification of receptor-ligand binding sites

Determination of the three-dimensional structure of the target protein either alone or even better in complex with the receptor-ligand by X-ray crystallography provides high-resolution and very high quality information about the molecular recognition of the

compound in the target protein structure. In the case, where the target is a soluble protein it is often possible to perform rationalized lead compound optimisation through crystallisation of the lead compound in complex with the target protein, analyse the molecular interactions and identify possible ways of improving these interactions and on this basis new compounds with improved affinity are synthesised. Subsequent X-ray analysis of complexes of these improved compounds and the target protein can then lead to the synthesis of a new series of further improved compounds, new compound-target crystallisations and so on until the desired affinity has been obtained.

However, these methods of structure based lead compound optimisation or "rational drug discovery" can only be applied to soluble proteins, which are relatively easy to crystallise. For example, membrane proteins, which constitute a majority of drug targets, are very difficult or in most cases still impossible to crystallise. A variety of methods have been employed in order to characterize receptor-ligand-receptor interactions in proteins where three-dimensional structures cannot be obtained. For example, site-directed mutagenesis is used to eliminate a receptor-ligand binding site or part of a receptor-ligand binding site by substitution of selected amino acid residues with other residues, e.g. alanine. Only a few cases have been presented where receptor-ligand binding sites have been thoroughly investigated by an extensive and systematic mutational analysis of all possible residues in a given area and with combination of both mutational analysis of the receptor and chemical analysis of the receptor-ligand (e.g. the β-adrenergic receptor, Strader et al., *FASEB J. 3*, 1989, pp. 1825-1832; Strader et al, *J. Biol. Chem. 266*(1), 1991, pp. 5-8; Schambye et al. *Mol. Pharm.*, 1995 47:425-431).

A general problem of the site-directed mutagenesis method is that it is not clear whether the substitution of a residue affects the binding of a receptor-ligand directly (i.e. the residue is directly involved in receptor-ligand binding) or indirectly (i.e. the residue is only involved in the structure of the receptor). Another problem of Ala substitution is false negative results because the procedure basically creates another "hole" in the presumed binding pocket through removal of the side chain on the residue replaced by Ala. The effect of Ala substitution is highly dependent on the relative contribution to the binding energy of the replaced residue. An alternative to Ala substitution is steric hindrance mutagenesis where for example a larger side chain, e.g. Trp, is introduced in a presumed binding pocket as described by Holst et al., *Mol Pharmacol. 53*(1), 1998, pp. 166-175.

Methods such as photoaffinity labelling has also been proven to be a useful tool in identifying domains of receptors involved in receptor-ligand binding (Dohlman et al., *Ann.*

Rev. Biochem. 60, 1991, pp. 653-688). A photoreactive group is attached or built into the receptor-ligand. After binding, the receptor-ligand-receptor complex is exposed to UV light, resulting in cross-linking of the receptor-ligand to the receptor. Finally the complex is digested with proteases and the receptor-ligand-binding part of the receptor can be identified.

It should be noted, however, that except for proteins where crystal- or NMR-structures can be made, it is only in a few cases where binding pockets for receptor-ligands in fact have been identified with a reasonable degree of accuracy. This is especially the case for membrane proteins. In even fewer cases have the actual pattern of chemical recognition been determined well in these proteins, i.e. identification of which chemical moiety of the receptor-ligand interacts with which side-chain or with which part of the backbone in the target (Schwartz et al. *Current Opin. Biotechnol.*, 1994 4:434-444). In the very few cases of for example membrane proteins where some information is available concerning the presumed binding pocket or perhaps even about actual chemical interactions, this is only the case for final, high-affinity optimised drugs. No information along these lines are today known for lead compounds found by chemical screening in for example membrane proteins. Even in the case where an X-ray structure is known for a complex between a compound or a drug and its target protein, it is often not possible to predict the binding mode of close analogues of this since modification of the compound may seriously alter the overall binding mode involving also parts of the compound which have not been chemically modified (Mattos et al. *Struct. Biol*, 1995 1:55-58). Thus, a chemical "anchor", i.e. a well identified binding point between a chemical moiety in the compound and a particular site in the target protein, would be highly beneficial in order to efficiently apply structure based drug discovery techniques to both proteins with known three dimensional structures and to protein targets for which meaningful molecular models can be built based on homology to known protein structures.

The present invention deals with libraries of chemical compounds useful in methods involving a chemical "anchor". Such methods make use of a metal-ion binding site in the target biological molecule as well a metal binding site in a chemical compound. The metal-ion binding site in the biological target molecule such as, e.g., a target protein may be a natural metal-ion binding site or it may be a metal-ion binding site that has been introduced into the protein by artificial means such as, e.g., engineering means.

Background of the invention

Natural metal-ion sites in proteins

Many proteins contain metal-ion binding sites. These metal-ion sites serve either structural purposes, for example stabilizing the three-dimensional structure of the protein, or they serve functional purposes, where the metal-ion may for example be part of the active site of an enzyme. It is well known that also several integral membrane proteins include binding sites for metal ions. The coordination of metal ions to metal ion binding sites is well characterized in numerous high-resolution X-ray and NMR structures of soluble proteins; for example, distances from the chelating atoms to the metal ion as well as the preferred conformation of the chelating side chains are known (e.g. J.P. Glusker, *Adv. Protein Chem. 42*, 1991, pp. 3-76; P. Chakrabarty, *Protein Eng. 4*, 1990, pp. 57-63; R. Jerigan et al., *Curr. Opin. Struct. Biol. 4*, 1994, pp. 256-263). Thus, metal-ion binding in proteins is one of the most well characterised forms of receptor-ligand-protein interactions known. Hence, characterising a metal ion-binding site in a membrane protein using, for example, molecular models and site directed mutagenesis can yield information about the structure of the membrane protein and importantly where the "ligand" (metal ion) binds (e.g. Elling et al. *Fold. Des.* 2(4), 1997, pp. S76-80).

Metal-ion site engineering in proteins

Engineering of artificial metal-ion binding sites into membrane proteins has been employed to explore the structure and function of these proteins. Thus, C.E. Elling et al., *Nature 374*, 1995, pp. 74-77, have reported how the binding site for a prototype antagonists for the tachykinin NK-1 receptor could be converted into a metal ion-binding site by systematic substitution of residues in the binding pocket with His residues. If side chains of amino acid residues participating in metal ion binding are known, it imposes a distance constraint on the protein structure which can be used in the interpretation of unknown protein structures (C.E. Elling and T.W. Schwartz, *EMBO J. 15*(22), 1996, pp. 6213-6219; C.E. Elling et al., *Fold. Des.* 2(4), 1997, pp. S76-80). Recently the generation of an activating metal-ion binding site has been reported for the β_2-adrenergic receptor, where the binding site for the normal catecholamine ligands was exchanged with a metal-ion site through specific substitutions in the binding pocket for the agonists (C.E. Elling et al, *PNAS 96*, 1999, pp. 12322-12327). This metal-ion binding site could be addressed also with metal-ions in complex with metal-ion chelators, i.e. small organic compounds binding metal-ions.

However, none of the above-mentioned documents address the concept of using a chemical "anchor" in the drug discovery process.

Summary of the invention

The present invention provides libraries of chemical compounds that are suitable for use in a molecular approach for rapidly and selectively identifying small organic molecule receptor-ligands, i.e. compounds that are capable of interacting with and binding to specific sites on biological target molecules. It has been possible to construct libraries with the aim of designing compounds specifically directed against predetermined epitopes on the biological target molecules. The compounds contained in a library of the invention are initially constructed to be bi-functional, i.e. having both a metal-ion binding moiety, which conveys them with the ability to bind to either a natural or an artificially constructed metal-ion binding site as well as a variable moiety, which is varied chemically to probe for interactions with specific parts of the biological target molecule located spatially adjacent to the metal-ion binding site. Compounds may subsequently be further modified to bind to the unmodified biological target molecule without help of the bridging metal-ion. The methods involving the use of a library according to the invention may be performed easily and quickly and lead to unambiguous results. The compounds identified by the methods described herein or in PCT/EP00/13389 may themselves be employed for various applications or may be further derivatised or modified to provide novel compounds.

The present invention further relates to the use of specific metal-ion binding sites in biological target molecules.

The libraries of the present invention are applicable in drug discovery processes involving any biological target molecule that has or can be manipulated to have a metal-ion binding site. However, in the following proteins are used as examples of biological target molecules.

Parts of the present invention utilise the finding that many proteins in their natural form possess a metal-ion binding site, which may or may not have been recognized previously. However, in order to obtain a general applicability of the technology to a broad range of biological target molecules, the invention especially utilises the possibility to mutate proteins, for example a receptor, an enzyme or a transcriptional regulator in such a way, that they comprise a metal-ion binding site. The metal-ion site is then used as an anchor-point for the initial parts of the medicinal chemistry drug-discovery process, during which

test compounds can be synthesized, which due to their specific interaction with the metal-ion binding site can be deliberately directed towards interaction with specific, functionally interesting parts of the biological target molecule.. The test compounds are subsequently structurally optimised for interaction with spatially neighbouring parts of the proteins (that is, interaction with the side chains or backbone of one or more neighbouring amino acid residues). These compounds can then be utilized as leads or starting points for the construction of receptor-ligands binding to the wild-type protein. In this way it is possible to predetermine the binding site of a compound to a particular location in a protein structure and thereby target the optimised compounds to sites where binding of the compound will alter the biological activity of the protein in a desired way, for example to increase or decrease its biological activity. By selecting the binding site for a test compound at will and thereby selecting the binding site for the optimised compound (such as a drug candidate) in a protein, it is for example possible to:

1) speed up the process of development of high affinity drug candidates or other compounds with biological activity because a more efficient structure-based compound optimisation process can be applied;
2) obtain high selectivity for a given member of a protein family by targeting the compound to a site in the protein which differs between different members of the protein family;
3) obtain new functionalities of compounds by targeting them to allosteric modulatory sites in proteins.

These constitute some of the advantages of the present invention.

In the course of research, the inventors have found that certain small organic compounds which bind metal ions (i.e. metal-ion chelators) are also able to bind to metal ion binding sites in various proteins, including membrane proteins for example receptors, in such a way that the metal ion acts as a bridge between the small organic compound and the protein. Importantly, the present invention has made it possible to use libraries of chemical compounds to predetermine or identify and localise the exact binding site and binding mode of such metal-ion chelates, contrary to what has been known in the art for test compounds in general. Based on the identification or confirmation of the binding site of the test compounds, using for example site-directed mutagenesis, three-dimensional structure determination by for example X-ray crystallography or NMR or molecular models of the protein and techniques such as those described above, a rational approach may be taken to the chemical optimisation of the test compounds. Thus, relatively small chemical

libraries may be made, the compounds in which may be designed to interact with specific amino acid residues of the protein in question. Compounds that exhibit a high affinity binding to the protein and affect the biological activity of the protein in a desired way may then be selected for further optimisation.

The metal-ion binding portion of the test compounds may subsequently be removed or altered to no longer posses metal-ion binding properties, and the test compounds, as well as chemical derivatives thereof may be constructed to interact with side chains of other amino acids in the vicinity of the artificial metal ion binding site, and tested for binding to the wild-type protein which does not include a metal ion binding site. Accordingly, relatively small chemical libraries may be made, the compounds in which may be designed to interact with the specific amino acid residues found in the wild-type protein at or spatially surrounding the location where the metal ion site had initially been engineered.

Thus, the present invention is based on the general principle, applicable to any biological target molecule including a protein, of introducing metal ion binding sites at any position in e.g. the protein where a test compound binding to the protein is likely to exert an effect on the biological activity of the protein. This may for example be 1) at a site where the test compound will interfere with the binding to another protein, for example a regulatory protein, or to a domain of the same protein; 2) at a site where the binding of the test compound will interfere with the cellular targeting of the protein; 3) at a site where the binding of the test compound will directly or indirectly interfere with the binding of substrate or the binding of an allosteric modulatory factor for the protein; 4) at a site where the binding of the test compound may interfere with the intra-molecular interaction of domains within the protein, for example the interaction of a regulatory domain with a catalytic domain; 5) at a site where binding of the test compound will interfere with the folding of the protein, for example the folding of the protein into its active conformation; or 6) at a site which will interfere with the activity of the protein, for example by an allosteric mechanism. Subsequent to identifying test compounds that bind to the artificial metal-ion binding site of the protein, information may be acquired of the structure of the binding site and of amino acid residues in its immediate vicinity. Such information may be used in the design of compounds with improved binding affinity to the proteins resulting from interaction with one or more amino acid residues in the vicinity of the metal-ion binding site. Such compounds may, in turn, be used in the design of potential drug candidates or other compounds with a desired activity on the corresponding wild-type, non-mutated protein.

Accordingly, the present invention relates to the use of a chemical library comprising three or more of a chemical compound in a drug discovery process for identification of a small organic compound that is able to bind to a biological target molecule. Such a drug discovery process comprises mutating a biological target molecule in such a way that at least one amino acid residue capable of binding a metal ion is introduced into the biological target molecule so as to obtain a metal ion binding site as an anchor point in the mutated biological target molecule.

The mutated biological target molecule may furthermore be contacted with a library of test compounds which comprises a moiety including at least two heteroatoms for chelating a metal ion, under conditions permitting non-covalent binding of the test compound to the introduced metal ion binding site of the mutated biological target molecule, and then followed by detection of any change in the activity of the mutated biological target molecule or determation of the binding affinity of the test compound to the mutated biological target molecule.

The present invention relates also to the use of a chemical library in a drug discovery process for identification of a small organic compound that is able to bind to a biological target molecule, which has at least one metal ion binding site. The drug discovery process comprises

(a) contacting the biological target molecule with a test compound which comprises a moiety including at least two heteroatoms for chelating a metal ion, under conditions permitting non-covalent binding of the test compound to the metal ion binding site of the biological target molecule, and

(b) detecting any change in the activity of the biological target molecule or determining the binding affinity of the test compound to the biological target molecule.

A very important class of biological target molecules amenable to testing according to the present invention are proteins such as membrane proteins, which includes proteins that are involved in intercellular communication and other biological processes of profound importance for cellular activity. Thus, in another aspect, the present invention relates to a chemical library suitable for use in a method of identifying a metal-ion binding site in a protein. Such a method comprises

(a) selecting a nucleotide sequence suspected of coding for a protein and deducing the amino acid sequence thereof,

(b) expressing said nucleotide sequence in a suitable host cell,

(c) contacting said cell or a portion thereof including the expressed protein with a test compound which comprises a moiety including at least two heteroatoms for chelating a metal ion, under conditions permitting non-covalent binding of the test compound to the protein, and detecting any change in the activity of the protein or determining the binding affinity of the test compound to the protein, and

(d) determining, based on the generic three-dimensional model of the class of proteins to which the protein or suspected protein belongs, at least one metal ion binding amino acid residue located in said protein to locate the metal ion binding site of said protein.

In a still further aspect the invention relates to chemical libraries for use in a method of mapping a metal-ion binding site of a protein. This method comprises

(a) contacting the protein with a library of test compounds, which compounds comprise a moiety including at least two heteroatoms for chelating a metal ion, under conditions permitting non-covalent binding of the test compound to the protein, and detecting any change in the activity of the protein or determining the binding affinity of the test compound to the protein, and

(b) determining, based on the primary structure of the specific protein in question and the generic three-dimensional model of the class of proteins to which the specific protein of step (a) belongs, at least one metal ion binding amino acid residue located in the membrane protein to identify the metal ion binding site of said membrane protein.

Accordingly, the invention relates to chemical libraries comprising test compounds in chelated or non-chelated form and to a chemical library comprising metal ions suitable for chelating test compounds. The metal ions are generally presented in salt form or in the form of complexes or solvates.

Further aspects of the invention as well as preferred embodiments of the invention appear from the appended claims.

The details and particulars described for libraries for use in e.g. the drug discovery process aspect apply mutatis mutandis – whenever relevant – to all other aspects of the invention.

Detailed description of the invention

Essential parts of the present invention relates to chemical libraries for use in methods of identifying compounds that are capable of binding to specific sites on biological target molecules. In a typical form of this process the following steps are involved:

(1) *Identification or engineering of metal-ion binding sites to be exploited as anchor points for lead compounds* - In one embodiment of the invention, the biological target molecule already has a suitable metal-ion site, which may or may not previously have been recognized. In another more broadly applicable form of the invention such metal-ion sites are introduced, for example through mutagenesis, at specific sites in the biological target molecule expected to be useful as anchor points for the development of compounds affecting the function of the target molecule in a desired way. In one form of the invention a number of such sites are introduced and one or more are selected for further use.

(2) *Selection of lead compound from library of metal-ion chelating compounds* - Basic libraries of metal-ion chelators exposing a systematic range of chemical moieties differing in potential chemical interaction-mode with the surrounding parts of the biological target molecule are screened for lead or test compounds which will bind to the metal-ion site in the biological target molecule and affect its function in a desired way.

(3) *Chemical optimisation of lead compound for secondary interaction points in the biological target molecule* - Based on the selected lead compound, libraries of basic bi- or multi-functional compounds are being constructed in which the compounds have both a anchoring *metal-ion binding moiety*, which conveys them with the ability to bind to the metal-ion binding site in the biological target molecule, as well as a *variable moiety*, which is varied chemically to probe for improved interactions with specific parts of the biological target molecule located spatially adjacent to the metal-ion binding site. In one preferred form of the invention these libraries are constructed based on structural knowledge of the chemical target moiety in the biological target molecule. In another form a more broad screening of larger libraries of compounds is performed without detailed knowledge of the structure of the biological target molecule surrounding the anchoring metal-ion site.

(4) *Chemical optimisation of lead compound for high affinity interaction with wild type biological target molecule – exchange of metal ion anchor with "ordinary" chemical interaction* - When a compound has been developed having a suitable, detectable affinity also on the wild-type form of the biological target molecule usually without metal-ion present, then this compound is further optimized for high affinity binding and effect on the wild-type molecule. In one form of the invention structure-based construction of chemical libraries will be performed in order to take advantage of the possibility to directly exchange the metal-ion bridge with other types of chemical interactions with the amino acid residues found in the wild type molecule.

The present invention is directed to chemical libraries for use in methods directly or indirectly involved in the above-mentioned drug discovery process. Furthermore, it is directed to the use of chemical libraries for selecting a chemical compound from a library.

The following detailed description of the invention is mainly concerned with methods of identifying compounds interacting with proteins such as, e.g., membrane proteins. It should be understood, however, that the discussion of the detailed method steps apply equally to other biological target molecules like nucleic acids, carbohydrates, nucleoproteins, glycoproteins and glycolipids. Furthermore, it should be understood that the chemical libraries of the present invention are suitable for use in such methods.

In the following some definitions are first dealt with following by a detailed description of the four main steps of the drug discovery process in which libraries of the present invention is employed.

Definitions

Throughout the text including the claims, the following terms shall be defined as indicated below.

A *"library"* is intended to indicate a collection of chemical compounds having a common basic structural element. The number of compounds in a library is three or more. All the chemical compounds contained in a library according to the invention have the same common basic structural element or scaffold. The number of compounds in a library is generally in a range of from about 3 to about 10,000 compounds such as, e.g. from about 5 to about 5,000, from about 10 to about 2,500, from about 3 to about 1,000, from about 3 to about 750, from about 3 to about 500, from about 3 to about 250, from about 3 to about

100, from about 3 to about 75, from about 3 to about 50, from about 3 to about 25, from about 3 to about 10 compounds. In general, libraries based on focused structures contain from about 3 to about 500 compounds such as, e.g. from about 3 to about 100 compound, whereas chemical diverse randomized libraries contain from about 500 to about 10,000 compounds such as, e.g. 750 to about 10,000 compounds, from about 1,000 to about 10,000 compounds.

A *"chemical compound"* or a *"test compound"* is intended to indicate a small organic molecule of low molecular weight or a small organic compound, which is capable of interacting with a biological target molecule, in particular with a protein, in such a way as to modify the biological activity thereof. The term includes in its meaning metal-ion chelates of the formulas shown below. Furthermore, the term includes in its meaning metal-ion chelates of the formulas shown below as well as chemical derivatives thereof constructed to interact with other part(s) of the biological target molecule than the metal-ion binding site. A chemical compound may also be an organic compound, which in its structure includes a metal atom via a covalent binding.

A *"metal-ion chelator"* is intended to indicate a chemical compound capable of forming a complex with a metal atom or ion, and contains at least two interactions between the metal centre and the chelator. Such a compound will generally contain two heteroatoms such as N, O, S, Se or P with which the metal atom or ion is capable of forming a complex.

A *"ligand"* is intended to indicate a functional group or a structural element that binds or coordinates a metal ion.

A *"metal-ion chelate"* is intended to indicate a complex of a metal ion chelator and a metal atom or ion.

A *"metal ion"* is intended to indicate a charged or neutral element. Such elements belong to the groups denoted main group metals, light metals, transition metals, semi-metals or lanthanides (according to the periodic system). The term "metal ion" includes in its meaning metal atoms as well as metal ions.

A *"metal-ion binding site"* is intended to indicate a part of a biological target molecule that comprises atoms in relative positions in such a way that they are capable of complexing with a metal atom or ion. Such atoms will typically be heteroatoms, in particular N, O, S,

Se or P. With respect to proteins a metal ion binding site is typically an amino acid residue of the protein which comprises an atom capable of forming a complex with a metal ion. These amino acid residues are typically, but not restricted to, histidine, cysteine, glutamate and aspartate.

A *"receptor-ligand"* is intended to include any substance that binds to a biological target molecule and thereby inhibiting or stimulating its activity. An "agonist" is defined as a ligand increasing the functional activity of a biological target molecule (e.g. signal transduction through a receptor). An "antagonist" is defined as a ligand decreasing the functional activity of a biological target molecule either by inhibiting the action of an agonist or by its own intrinsic activity. An "inverse agonist" (also termed "negative antagonist") is defined as a ligand decreasing the basal functional activity of a biological target molecule.

A *"biological target molecule"* is intended to include proteins such as, e.g., membrane proteins, nucleic acids, carbohydrates, nucleoproteins, glycoproteins and glycolipids. In the present context the biological target molecule may have has been manipulated to contain a metal-ion binding site. However, in some cases, the biological target molecule may be in its wild-type form.

A *"protein"* is intended to include any protein, polypeptide or oligopeptide with a discernible biological activity in any unicellular or multicellular organism, including bacteria, fungi, plants, insects, animals or mammals, including humans. Thus, the protein may suitably be a drug target, i.e. any protein which activity is important for the development or amelioration of a disease state, or any protein which level of activity may be altered (i.e. up- or down-regulated) due to the influence of a biologically active substance such as a small organic chemical compound.

A *"membrane protein"* is intended to include but is not limited to any protein anchored in a cell membrane and mediating cellular signalling from the cell exterior to the cell interior. Important classes of membrane proteins include receptors such as tyrosine kinase receptors, G-protein coupled receptors, adhesion molecules, ligand- or voltage-gated ion channels, or enzymes. The term is intended to include membrane proteins whose function is not known, such as orphan receptors. In recent years, largely as part of the human genome project, large numbers of receptor-like proteins have been cloned and sequenced, but their function is as yet not known. The present invention may be of use in elucidating the function of the presumed receptor proteins by making it possible of

identifying ligand for orphan receptors based on compounds developed from metal-ion chelates that bind to mutated orphan receptors into which artificial metal ion binding sites have been introduced.

"Signal transduction" is defined as the process by which extracellular information is communicated to a cell by a pathway initiated by binding of a ligand to a membrane protein, leading to a series of conformational changes resulting in a physiological change in the cell in the form of a cellular signal

A *"functional group"* is intended to indicate any chemical entity which is a component part of the chemical compound and which is capable of interacting with an amino acid residue or a side chain of an amino acid residue of the biological target molecule such as, e.g. a membrane protein. A functional group is also intended to indicate any chemical entity, which is a component part of the biological target molecule and which is capable of interacting with other parts of the biological target molecule or with a part of the chemical compound. Functional groups may be involved in interactions such as, e.g., ionic interactions, ion-dipole interactions, dipole-dipole interactions, hydrogen bond interactions, hydrophobic interactions, pi-stacking interactions, edge-on aromatic interactions, dispersion and induction forces or metal complex interactions.

A *"wild-type"* membrane protein is understood to be a membrane protein in its native, non-mutated form, in this case not comprising an introduced metal ion binding site

The term *"in the vicinity of"* is intended to include an amino acid residue or any other residue or functional group located in the space defined by the binding site of the metal ion chelate and at such a distance from the metal ion binding amino acid residue that it is possible, by attaching suitable functional groups to the chemical compound, to generate an interaction between said functional group or groups and said amino acid residue, another residue or functional group.

A *"linker"* is intended to embrace bifunctional chemical substances which on the one hand are able to react or interact with the test compound and on the other hand with a carrier.

A *"carrier"* is intended to embrace compounds that provides the test compounds with specific properties, e.g. with respect to the physiological, pharmacokinetic and/or biopharmaceutic behaviour of the test compound. The carrier is normally directly or indirectly linked to the test compound through establishment of a covalent or a non-

covalent bound. When the carrier is indirectly linked to the test compound, a spacer between the test compound and the carrier is normally included.

Nature of the biological target molecules

The biological target molecules include but are not restricted to proteins, nucleoproteins, glycoproteins, nucleic acids, carbohydrates, and glycolipids. In the present context the biological target molecule contains or has been manipulated to contain a metal-ion binding site. In preferred embodiments the biological target molecule is a protein, which may be for example a membrane receptor, a protein involved in signal transduction, a scaffolding protein, a nuclear receptor, a steroid receptor, a transciption factor, an enzyme, and an allosteric regulator protein, or it may be a growth factor, a hormone, a neuropeptide or an immunoglobulin.

Proteins as drug targets

Most drug compounds act by binding to and altering the function of proteins. These can be intracellular proteins such as, for example enzymes and transcription factors, or they can be extracellular proteins, for example enzymes, or they can be membrane proteins. Membrane proteins constitute a numerous and varied group whose function is either structural, for example being involved in cell adhesion processes, or the membrane proteins are involved in intercellular communication and communication between the cell exterior and the interior by transducing chemical signals across cell membranes, or they facilitate or mediate transport of compounds across the lipid membrane. Membrane proteins are for instance receptors and ion channels to which specific chemical messengers termed receptor-ligands bind resulting in the generation of a signal, which gives rise to a specific intracellular response (this process is known as signal transduction). Membrane proteins can, for example also be enzymes which are associated to the membrane for functional purposes, e.g. proximity to their substrates. Most membrane proteins are anchored in the cell membrane by a sequence of amino acid residues, which are predominantly hydrophobic to form hydrophobic interactions with the lipid bilayer of the cell membrane. Such membrane proteins are also known as integral membrane proteins. In most cases, the integral membrane proteins extend through the cell membrane into the interior of the cell, thus comprising an extracellular domain, one or more transmembrane domains and an intracellular domain.

A large fraction of current drugs act on membrane proteins and among these the majority are targeted towards the G protein coupled receptors (GPCR) with their seven transmembrane segments, also called 7TM receptors.

Membrane proteins as drug targets

In particularly preferred embodiments the biological target molecule is a membrane protein, which suitably is an integral membrane protein, which is to say a membrane protein anchored in the cell membrane. The membrane protein is preferably of a type comprising at least one transmembrane domain. Interesting membrane proteins for the present purpose are mainly found in classes comprising 1-14 transmembrane domains.

1TM - membrane proteins of interest comprising one transmembrane domain include but are not restricted to receptors such as tyrosine kinase receptors, e.g. a growth factor receptor such as the growth hormone, insulin, epidermal growth factor, transforming growth factor, erythropoietin, colony-stimulating factor, platelet-derived growth factor receptor or nerve growth factor receptor (TrkA or TrkB).

2TM - membrane proteins of interest comprising two transmembrane domains include but are not restricted to, e.g., purinergic ion channels.

3, 4, 5TM - membrane proteins of interest comprising 3, 4 or 5 transmembrane domains includes but are not restricted to e.g. receptor-ligand-gated ion channels, such as nicotinic acetylcholine receptors, GABA receptors, or glutamate receptors (NMDA or AMPA).

6TM - membrane proteins of interest comprising 6 transmembrane domains include but are not restricted to e.g., voltage-gated ion channels, such as potassium, sodium, chloride or calcium channels.

7TM — membrane proteins of interest comprising 7 transmembrane domains include but are not restricted to G-protein coupled receptors, such as receptors for (– in brachet the receptor subtypes are mentioned): acetylcholine (m1-5), adenosine (A1-3) and other purines and purimidines (P2U and P2Y1-12), adrenalin and noradrenalin (α1A-D, α2A-D and β1-3), amylin, adrenomedullin, anaphylatoxin chemotactic factor, angiotensin (AT1A, -1B and -2), apelin, bombesin, bradykinin (1and 2), C3a, C5a, calcitonin, calcitonin gene related peptide, CD97, conopressin, corticotropin releasing factor (CRF1and -2), calcium, cannabinoid (CB1and -2), chemokines (CCR1-11, CXCR1-6, CX3CR and XCR),

cholecystokinin (A-B), corticotropin-releasing factor (CRF1-2), dopamine (D1-5), eicosanoids, endothelin (A and B), fMLP, Frizzled (Fz1,2,4,5 and 7-9), GABA (B1 and B2), galanin, gastrin, gastric inhibitory peptide (glucose-dependent insulinotropic polypeptide), glucagon , glucagon-like peptide I and II, glutamate (1-8), glycoprotein hormone (e.g. FSH, LSH, TSH, LH), growth hormone releasing hormone, growth hormone secretagogue /Ghrelin, histamine (H1-4), 5-hydroxytryptamine (5HT1A-1F, -2A-C and -4-7), leukotriene, lysophospholipid (EDG1-4), melanocortins (MC1-5), melanin concentrating hormone (MCH 1 and 2), melatonin (ML1A and 1B), motilin, neuromedin U, neuropeptide FF (NFF1and 2), neuropeptide Y (NPY1,2,4,5 and 6), neurotensin (1 and 2), nocioceptin, odor components , opiods (κ, δ, μ and x), orexins(OX1and -2) , oxytocin, parathyroid hormone/parathyroid hormone-related peptides, pheromones, platelet-activating factor, prostaglandin (EP1-4 and F2) prostacyclin, pituitary adenylate activating peptide, retinal, secretin, smoothemd, somatostatins (SSTR1-5), tachykinins (NK1-3), thrombin and other proteases acting through 7TM receptor, thromboxane, thyrotropin-releasing hormone, vasopressin (V1A, -1B and -2), vasoactive intestinal peptide, urotensin II, and virally encoded receptors (US27, US28, UL33, UL78, ORF74, U12, U51); and 7TM proteins coded for in the human genome but for which no endogenous ligand has yet been assigned such as mas-proto-oncogene, EBI (I and II), lactrophilin, brain specific angiogenesis inhibitor (BAI1-3), EMR1, RDC1 receptor, GPR12 receptor or GPR3 receptor, and 7TM proteins coded for in the human genome but for which no endogenous ligand has yet been assigned.

8, 9, 10, 11, 12, 13, 14TM - Membrane proteins of interest comprising 8 to 14 transmembrane domains include but are not restricted to e.g., transporter proteins, such as a GABA, monoamine or nucleoside transporter.

The membrane protein may also be a multidrug resistance protein, e.g. a P-glycoprotein, multidrug resistance associated protein, drug resistance associated protein, lung resistance related protein, breast cancer resistance protein, adenosine triphosphate-binding cassette protein, Bmr, QacA or EmrAB/TolC pump.

The membrane protein may also be a cell adhesion molecule, including but not restricted to for example NCAM, VCAM, ICAM or LFA-1.

Furthermore, the membrane protein may be an enzyme such as adenylyl cyclase.

7TM overview

In a particularly preferred embodiment of the invention, the biological target molecules are 7 transmembrane domain receptors (7TM receptors) also known as G-protein coupled receptors (GPCRs). This family of receptors constitutes the largest super-family of proteins in the human body and a large number of current drugs are directed towards 7TM receptors, for example: antihistamines (for allergy and gastric ulcer), beta-blockers (for cardiovascular diseases), opioids (for pain), and angiotensin antagonists (for hypertension). These current drugs are directed against relatively few receptors, which have been known for many years. Today, several hundred 7TMs have been cloned and characterized, and the total number of different types of 7TMs in humans is presumed to be between 1 and 2.000. The spectrum of receptor-ligands acting through 7TMs includes a wide variety of chemical messengers such as ions (e.g. calcium ions), amino acids (glutamate, γ-amino butyric acid), monoamines (serotonin, histamine, dopamine, adrenalin, noradrenalin, acetylcholine, cathecolamine, etc.), lipid messengers (prostaglandins, thromboxane, anandamide, etc.), purines (adenosine, ATP), neuropeptides (tachykinin, neuropeptide Y, enkephalins, cholecystokinin, vasoactive intestinal polypeptide, etc.), peptide hormones (angiotensin, bradykinin, glucagon, calcitonin, parathyroid hormone, etc.), chemokines (interleukin-8, RANTES, etc.), glycoprotein hormones (LH, FSH, TSH, choriogonadotropin, etc.) and proteases (thrombin). It is expected that a large number of the members of the 7TM superfamily of receptors will be suitable as drug targets. This notion is based on the fact that these receptors are involved in controlling major parts of the chemical transmission of signals between cells both in the endocrine and the paracrine system in the body as well as within the nervous system.

7TM receptor signalling - In 7TMs, binding of the chemical messenger to the receptor leads to the association of an intracellular G-protein, which in turn is linked to a secondary messenger pathway. The G-protein consists of three subunits, an α-subunit that binds and hydrolyses GTP, and a $\beta\gamma$-subunit. When GDP is bound, the α subunit associates with the $\beta\gamma$ subunit to form an inactive heterotrimer that binds to the receptor. When the receptor is activated, a signal is transduced by a changed receptor conformation that activates the G-protein. This leads to the exchange of GDP for GTP on the α subunit, which subsequently dissociates from the receptor and the $\beta\gamma$ dimer, and activates downstream second messenger systems (e.g. adenylyl cyclase). The α subunit will activate the effector system until its intrinsic GTPase activity hydrolyses the bound GTP to GDP, thereby inactivating the α subunit. The $\beta\gamma$ subunit increases the affinity of the α subunit for GDP but may also be directly involved in intracellular signalling events.

7TM receptor-ligand-binding sites - Mutational analysis of 7TMs has demonstrated that functionally similar but chemically very different types of receptor-ligands can apparently bind in several different ways and still lead to the same function. Thus monoamine agonists appear to bind in a pocket relatively deep between TM-III, TM-V and TM-VI, while peptide agonists mainly appear to bind to the exterior parts of the receptors and the extracellular ends of the TMs (Strader et al., (1991) J. Biol. Chem. 266: 5-8; Strader et al., (1994) Ann. Rev. Biochem. 63: 101-132; Schwartz et al. Curr. Pharmaceut. Design. (1995), 1: 325-342). Moreover, receptor-ligands can be developed independent on the chemical nature of the endogenous receptor-ligand, for example non-peptide agonists or antagonists for peptide receptors. Such non-peptide antagonists for peptide receptors often bind at different sites from the peptide agonists of the receptors. For instance, non-peptide antagonists may bind in the pocket between TM-III, TM-V, TM-VI and TM-VII corresponding to the site where agonists and antagonists for monoamine receptors bind (Holst et al. (1998) Mol. Pharmacol. 53:166-175). It has been found that in the substance P receptor, when the binding site for a non-peptide antagonist has been exchanged for a metal-ion binding site through introduction of His residues, no effect on agonist binding was observed (Elling et al., (1995) Nature 374: 74-77; Elling et al. (1996) EMBO J. 15: 6213-6219). It is believed that the non-peptide antagonist and the zinc ions act as antagonists by selecting and stabilizing an inactive conformation of the receptor that prevents the binding and action of the agonist. This illustrates that drugs can be developed totally independent on knowledge of the endogenous ligand, since there need not be any overlap in their binding sites.

Generic numbering system for 7TMs – The 7TM receptor superfamily is composed many hundreds of receptors that may be further divided into smaller sub-families of receptors. The largest of these smaller sub-families of 7TM receptors is composed of the rhodopsin-like receptors (also termed the family A receptors), which are named after the light-sensing molecule from our eye. The receptors are integral membrane proteins characterized by seven transmembrane (7TM) segments traversing the membrane in an antiparallel way, with the N-terminal on the extracellular side of the membrane and the C-terminal on the intracellular side. Within the membrane embedded part and in some cases in the membrane proximal parts, embedded in the aqueous environment surrounding the cell membrane, the polypeptide adopts a helical secondary structure. The lengths, and the beginning center and ends relative to the lipid bilayer membrane of these helices may be deduced from solved three-dimensional structures of the receptor proteins (Palczewski K. et al., Science, 289(5480), 2000, pp. 739-45). However, since the three-dimensional structure of only a single receptor has been solved to date, the helical lengths, and the

beginning, center and ends relative to the lipid bilayer membrane of each of the seven helices may be dissected by sequence analysis (J.M. Baldwin, EMBO J. 12(4), 1993, pp. 1693-703; J.M. Baldwin et al., J. Mol Biol, 272(1), 1997, pp. 144-64).

A useful tool in the identification and engineering of metal-ion sites is the generic numbering system for residues of 7TM receptors. The largest family of 7TM receptors is composed of the rhodopsin-like receptors, which are named after the light-sensing molecule from our eye. Within the many hundred members of the rhodopsin-like receptor family, a number of residues, termed key residues, especially within each of the transmembrane segments are highly but not totally conserved. These residues may be used to direct an alignment of the primary protein sequences within the transmembrane segments together with other standard principles and techniques, for example hydrophobicity plots, well-known to persons skilled in the art. Additionally, a number of other residues occur within the transmembrane segments that are very conserved, and these may be used to further direct an alignment of the transmembrane segments. These are particularly useful when a given key residue in a transmembrane segment has been substituted through evolution by another aminoacid of a dissimilar physiochemical nature.

However, due to differences in the length of especially the N-terminal segment, residues located at corresponding positions in different 7TM receptors are numbered differently in different receptors. However, based on the conserved key residues in each TM, a generic numbering system has been suggested (JM Baldwin, EMBO J. 12(4), 1993, pp. 1693-1703; TW Schwartz, Curr. Opin. Biotech. 5, 1994, pp. 434-444).
On the basis of the key residues present in the receptor family, the transmembrane segments are generically numbered. For example, in TM-II the highly conserved acidic function, aspartate (Asp) in the rhodopsin-like family is given the generic number 10, i.e. AspII:10, on the basis of its position in the helix. All other residues in the helix are hence numbered on this basis.

In Fig. 1 a schematic depiction of the structure of rhodopsin-like 7TMs is shown with one or two conserved, key residues highlighted in each TM: AsnI:18; AspII:10; CysIII:01 and ArgIII:26; TrpIV:10; ProV:16; ProVI:15; ProVII:17. In relation to the present invention it is important that residues involved in for example metal-ion binding sites can be described in this generic numbering system. For example, a tri-dentate metal-ion site constructed in the tachykinin NK1 receptor (Elling et al., (1995) Nature 374, 74-77) and subsequently transferred to the kappa-opioid receptor (Thirstrup et al., (1996) J. Biol. Chem. 271, 7875-7878) and to the viral chemokine receptor ORF74 (Rosenkilde et al., J. Biol. Chem. 1999

Jan 8; 274(2), 956-61) can be described to be located between residues V:01, V:05, and VI:24 in all of these receptors although the specific numbering of the residues is very different in each of the receptors. It is only in the rhodopsin-like receptor family that a generic numbering system has been established; however, it should be noted that although the sequence identity between the different families of 7TM receptors is very low, it is believed that they may share a more-or-less common seven helical bundle structure.

Hence an analogous system may be developed for the other families of 7TM receptors, for example the family B class of receptors, composed of for example the glucagon receptor, the glucagon-like peptide (GLP) receptor-1, Gastric inhibitory peptide receptor (GIP), the corticotropin releasing factor (CRF) receptor-1, vasoactive intestinal peptide (VIP) receptor, pituitary adenylate cyclase-activating polypeptide (PACAP) receptor etc. (J.W. Tams et al., Receptors Channels 1998;5(2), pp. 79-90). Again, on the basis of the key residues present in the family B class of receptors, the transmembrane segments are generically numbered. For example, in TM-I, the highly conserved hydroxy function, serine (Ser) is given the generic number 8, i.e. SerI:08 on the basis of its position in the helix; in TM-II the highly conserved histidine (His) is given the generic number 6 i.e. HisII:06; in TM-III the highly conserved cysteine (Cys) is given the generic number 1 i.e. CysIII:01; in TM-IV the highly conserved proline (Pro) is given the generic number 13 i.e. ProIV:13; in TM-V the highly conserved asparagine (Asn) is given the generic number 14 i.e. AsnV:14; in TM-VI the highly conserved leucine (Leu) is given the generic number 9 i.e. LeuVI:09; in TM-VII the highly conserved glycine (Gly) is given the generic number 13 i.e. GlyVII:13. All other residues in the helices are hence numbered on this basis.

Thus, all the techniques described in the present invention can be applied to the other families of 7TM receptors with minor modifications. This generic numbering system together with general knowledge of the 3D structure of the 7TM receptors and knowledge from systematic metal-ion site engineering makes it possible to predict or identify the presence of metal-ion sites based on the DNA sequence coding for the 7TM receptor.

Orphan 7TM receptors – one embodiment of the invention is directed to a method of developing assay for orphan 7TM receptors by the introduction of metal-ion sites in the orphan receptor. During the cloning of 7TM receptors many "extra" receptors were discovered for which no receptor-ligand was known, the so-called orphan receptors. Today there are several hundreds of such orphan 7TM receptors. Based on characterization of their expression pattern in different tissues or expression during development or under particular physiological or patho-physiological conditions and based

on the fact that the orphan receptors sequence-wise appear to belong to either established sub-families of 7TM receptors or together with other orphans in new families, it is believed that the majority of the orphan receptors are in fact important entities. As stated by representatives from the big pharmaceutical companies: Orphan 7TMs are "the next generation of drug targets" or "A neglected opportunity for pioneer drug discovery" (Wilson et al. Br.J.Pharmacol. (1998) 125: 1387-92; Stadel et al. Trends Pharmacol.Sci. (1997) 18: 430-37). Over the years receptor-ligands have been discovered for some of the orphan 7TM receptors, which then immediately have been recognized as "real" drug targets, for example: nocioceptin (for pain) (Reinscheid et al. Science (1995) 270: 792-94), orexin (for appetite regulation and regulation of energy homeostasis) (Sakurai et al. Cell (1998) 92: 573-85), melanin-concentrating hormone (for appetite regulation) (Chambers et al. Nature (1999) 400: 261-65), and cysteinyl leukotrienes (inflammation, especially asthma) (Sarau et al. Mol. Pharmacol. (1999) 56: 657-63). In the latter case, a number of drugs (for example pranlukast, zafirlukast, montelukast, pobilukast) had in fact been developed in recent years against the receptor as a physiological entity without having access to the cloned receptor – which turned out to be a "well known" orphan receptor. The problem is that it is very difficult to characterize orphan receptors and find their endogenous receptor-ligands, since no assays are available for these receptors due to the lack of specific receptor-ligands – a "catch 22" situation. The present invention is aimed at eliminating this problem. By introducing metal ion binding sites in orphan receptors at locations where it is known from previous work on multiple other 7TM receptors with known receptor-ligands and with binding and functional assays that binding of metal-ions and metal ion chelates will act as either agonists or more common as antagonists, then it will be possible to establish binding assays and functional assays for the orphan receptors. Binding of metal-ion chelates can be monitored either through functional assays in cases where agonistic metal ion sites are created, or through receptor-ligand binding assays. For example, many aromatic metal-ion chelators are by themselves fluorescent and can therefore directly be used as tracers in binding assays. Or, radioactive or other measurable indicators can be incorporated into the metal-ion chelator. By establishing a metal-ion chelator based receptor analysis for the orphan receptors, it will become possible to search for the elusive endogenous receptor-ligands or it will be possible to use the orphan receptors in various forms for drug discovery technology, for example high throughput screening. It should be noted that due to the initial lack of knowledge of the endogenous receptor-ligand and therefore also lack of knowledge of the binding site for this receptor-ligand in the 7TM receptor, there is a certain danger that the introduced metal ion binding site can interfere with receptor-ligand binding or signal transduction. However, based on metal-ion site engineering in multiple

7TM receptors and on mutational mapping of binding sites in multiple 7TM receptors, it will be possible to introduce such metal-ion sites at different locations in the receptor in an attempt to eliminate this problem. Moreover, an artificial binding site and binding analysis, which may interfere with the binding of the natural receptor-ligand, may still be useful for screening for receptor-ligands, for example antagonists.

Source of the biological target molecules

The biological target molecules of interest may be obtained in a useful form by different ways including but not limited to recombinantly, synthetically or commercially.

Cloning and expression - In a preferred embodiment the biological target molecule being a protein is obtained recombinantly. This can be achieved through cloning of the gene for the protein from genomic or cDNA libraries generally by the use of PCR techniques in accordance with standard techniques (eg. Sambrook et al. Molecular Cloning: A laboratory manual, 2. Ed. Cold Spring Harbor Laboratory, New York 1989), and expression of the gene in a suitable cell. The nucleotide sequence encoding the target protein - and mutant versions thereof (see below) - may be inserted into a suitable expression vector for the purpose of expression and analysis in a host organism. Thus regulatory element ensuring either constitutive or inducible expression of the protein of interest should be present in the vector, including promoter elements. The host organism into which the nucleotide sequence is introduced may be any cell type or cell line, which is capable of producing the target molecule in a suitable form for the test to be performed including but not restricted to e.g. yeast cells and higher eukaryotic cells such as e.g. insect or mammalian cells. Transformation of the cell line of choice may be performed by standard techniques routinely employed in the field as described e.g.. in Wigler et al. Cell (1978) 14: 725 and in accordance with standard techniques (Sambrook et al. Molecular Cloning: A Laboratory Manual, 2. ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). In a particularly preferred embodiment the biological target molecule being a membrane protein is expressed and tested in mammalian cells usually within the membrane and usually in whole cells or in isolated membrane preparations, which is dealt with and described further in the examples presented in "EXPERIMENTAL". Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650 and 1651), BHK (ATCC CRL 1632, ATCC CCL 10), CHL (ATCC CCL39), CHO (ATCC CCL 61), HEK293 (ATCC CRL 1573) and NIH/3T3 (ATCC CRL 1658) cell lines.

Isolation and purification - In the case where the biological target molecules is a soluble protein, for example an enzyme, a preferred source may be recombinantly produced protein, which subsequently is isolated and purified to a suitable purity and in a form suited for functional testing by various standard protein chemistry methods well known to those skilled in the art.

Functional testing of the biological target molecules

As part of the drug discovery process of the current invention, the biological target molecule comprising a natural or an engineered metal-ion binding site is contacted with a test compound for example consisting of a metal-ion in complex with a metal-ion chelator and any change in the biological activity of the biological target molecule is detected or the binding affinity of the test compound is determined.

Due to the diversity of biological target molecules, a wide variety of functional test can be performed depending on the individual target molecule and its functions. For example, for a soluble enzyme a suitable enzymatic analysis could be used on the purified enzyme (as described for Factor VIIa). For certain transcription factors a suitable gene-expression reporter assay could, for example be performed in a whole cell preparation. In a preferred embodiment of the invention the biological target molecule is a membrane protein and the effect of test compounds is monitored on the signal transduction process of the receptor, i.e. its ability to influence intracellular levels of for example cAMP, inositol phosphates, calcium mobilization etc. in response to the natural receptor-ligand. For instance, in the case of a 7TM receptor, this may entail the effect on signalling mediated through the intracellular G-protein. In this way, the testing may reveal whether the binding of a metal-ion (complex) may affect the activity of the target in for instance an antagonistic or an agonistic fashion. For the most part tests are performed as dose-response analysis in which a range of concentration of metal-ion chelator complexes are exposed to the biological target molecule.

When appropriate, the binding affinity of the test compound to the biological target molecule is determined, for example in competition binding experiments against a suitable radioactively labelled receptor-ligand for the protein target. Or, the affinity of the test compound can in some cases be determined by use of a chelating agent, which in itself is detectable or which can be labelled with a detectable labelling agent.

Structural testing of the biological target molecule

In a preferred embodiment of the invention, the 3D structure of the test compound in complex with the biological target molecule is determined, for example by techniques such as X-ray analysis of crystals of the receptor-ligand-protein complex or, for example by nuclear magnetic resonance (NMR) spectroscopic analysis of complexes in solution – all known to those skilled in the art. In this way the amino acid residues located in the vicinity of the metal-ion site and the chemical interaction of the bifunctional test compound with specific residues in the biological target molecule can be determined as control and as basis for the structure-based design of further modifications of the lead test compound and design of new libraries of compounds. Further, the effect of the test compound on the structure of the biological target protein, domains of this and or effect on the interaction of the target protein with other proteins can be determined.

Identification of metal-ion sites in biological target molecules

In a preferred embodiment of the present invention, naturally occurring metal-ion sites are used as initial attachment sites for metal-ion chelating test compounds in the drug discovery process. In general, such natural metal-ion sites can be identified functionally by studying the effect of either free metal-ions or by the effect of a library of metal-ion chelator complexes on any function of the biological target molecule. Metal-ion sites can also be identified or confirmed by structural means as described above and location of the site can also be identified by careful, controlled mutagenesis, i.e. exchanging of the residues involved in metal-ion binding with residues not having this property. Natural metal-ion sites are interesting drug targets since binding of a drug at or close by a natural metal-ion site often will act as an allosteric agent, i.e. affecting the structure and function of the biological target molecule at a site different from the usual active site, where most receptor-ligands will bind and act (see below).

Natural metal-ion sites in proteins in general - Metal-ion sites are known to occur in many biological target molecules including but not restricted to, for example proteins, glycoproteins, RNA, etc. These sites can serve either structural or functional purposes. Some metal-ion sites are known to occur solely from functional data, for example Zn(II)-sites in receptor-ligand gated ion channels. Or previously unknown metal-ion sites are discovered in the crystal structure of the protein, as for example Zn(II) sites in rhodopsin. Independent on the physiological purpose of the naturally occurring metal-ion site they may be targeted by the technology of the present invention, where they are addressed not

only by a metal-ion, but by a metal-ion in complex with a metal-ion chelator, which can affect the protein structure and function differently than the free metal-ion.

Natural metal-ion sites in 7TM receptors - naturally occurring metal ion sites have been described in two 7TM receptors: the tachykinin NK3 receptor (Rosenkilde et al. (1998) FEBS Lett. 439: 35-40) and the galanin receptor (Kask et al. (1996) EMBO J. 15: 236-240). In the NK3 receptor Zn(II) was shown to act as an enhancer (positive modulator) for agonist binding and action without itself being an agonist. Through mutagenesis the metal ion binding site was mapped to residues V:01 and V:05 at the extra-cellular end of TM-V. In the galanin receptor Zn(II) was shown to act as an antagonist for galanin binding, but the site was not characterized further. However, based on knowledge from metal-ion site engineering in 7TM receptors in general (see below) it is possible based on sequence analysis and molecular models to find previously unnoticed metal-ion sites in 7TM receptors. Some of these sites, for example the known one in the NK3 receptor, may be affected physiologically by free metal ions, for example when a receptor is expressed in brain regions where extra-cellular zinc concentrations may vary around 10^{-5} molar. However, many of the previously unnoticed metal ion sites may just be a reflection of the fact that polar, metal-ion binding amino acid residues (for example: His, Cys, Asp etc.) frequently are found in the water-exposed main receptor-ligand-binding crevice of 7TM receptors. In one embodiment of the present invention, these residues are used as initial attachment sites for metal ion chelating test compounds, i.e. lead compounds in the drug discovery process.

Engineering of metal-ion sites in biological target molecules

It is generally known that metal-ion sites can be built into proteins by introduction of metal-ion chelating residues at appropriate sites. In a particularly preferred embodiment of the invention such sites are constructed at strategic sites in the biological target molecule with the purpose to serve as anchor sites for test compounds in a drug discovery process and thereby target the medicinal chemistry part of the process towards particularly interesting epitopes on the target molecule.

Mutagenesis - the nucleotide sequence encoding the target protein of interest may be subjected to site-directed mutagenesis in order to introduce the amino acid residue, which includes the metal-ion binding site. Site-directed mutagenesis may be performed according to well-known techniques, e.g. as described in Ho et al. Gene (1989) 77: 51-59. In a specific, non-limiting example the mutation is introduced into the coding sequence of

the target molecule by the use of a set of overlapping oligonucleotide primer both of which encode the mutation of choice and through polymerisation using a high-fidelity DNA polymerase such as e.g. Pfu Polymerase (Stratagene) according to manufacturers specifications. The presence of the site-directed mutation event is subsequently confirmed through DNA sequence analysis throughout the genetic segment generated by PCR. In order to generate a metal-ion binding site this may involve the introduction of one or more amino acid residues capable of binding metal-ions including but not restricted to, for example His, Asp, Cys or Glu residues.

Generally the mutated target molecule will initially be tested with respect to the ability to still constitute a functional, although altered, molecule through the use of an activity assay suitable in the specific case. It should be noted that although mutations in proteins may obviously occasionally alter the structure and affect the function of the protein, this is by far always the case. For example, only a very small fraction (less than ten) of the many hundred Cys mutations performed in rhodopsin as the basis for site directed spin-labelling experiments and in for example the dopamine and other 7TM receptors as the basis for Cys accessibility scanning experiments have impaired the function of these molecules. Similarly, in the bacterial transport protein Lac-permease almost all residues have been mutated and only a few of these substitutions directly affect the function of the protein. Mutations will often also be performed in the biological target molecule to confirm or probe for the chemical interaction of test compounds with other residues in the vicinity of the natural or the engineered metal-ion site often as an integrated part of the general drug discovery process.

Metal-ion site engineering in protein targets in general - The drug discovery process may suitably include a step of determining the location of, for example the metal ion binding amino acid residue(s) in a mutated protein and determining the location of at least one other amino acid residue in the vicinity of the metal ion binding amino acid residue, based on either the actual three-dimensional structure of the specific biological target molecule in question (e.g. by conventional X-ray crystallographic or NMR methods) or based on molecular models based on the primary structure of the specific molecule together with the three-dimensional structure of the class of molecules to which the specific molecule belongs (e.g. established by sequence homology searches in DNA or amino acid sequence databases).

In the biological target molecule, the metal-ion binding site may suitably be introduced to serve as an anchoring, primary binding site for the test compound, which can thereby be

targeted to affect a site in the biological target molecule having one or more of the following properties (the metal-ion site may be placed either within or close to this site):

a site where the biological target molecule binds to another biological target molecule, for example a regulatory protein,

a site which will control the activity of the biological target molecule in a positive or negative fashion (i.e. up-regulating or down regulating the activity of the biological target molecule), for example by an allosteric mechanism,

a site where the binding of the test compound will directly or indirectly interfere with the binding and/or the function of the substrate or natural receptor-ligand or the binding of an allosteric modulatory factor for the biological target molecule,

a site where the binding of the test compound may interfere with the intra-molecular interaction of domains within the biological target molecule, for example the interaction of a regulatory domain with a catalytic domain,

a site where binding of the test compound will interfere with the folding of the biological target molecule, for example the folding of a protein into its active conformation,

a site where the binding of the test compound will interfere with the cellular targeting of the biological target molecule,

a site where the binding of the test compound will stabilise a conformation of the biological target molecule, which presents an epitope normally involved in protein-protein interactions in a non-functional form.

This list of properties is by no means exhaustive and only serves to give some examples of the possibilities which can be obtained by targeting the test compound and thereby the final drug candidate to specific epitopes in the biological target molecule through the drug discovery process of the present invention.

This will potentially provide the receptor-ligand with other pharmacological properties than agents normally acting at the active site. It is for example likely that compounds binding at allosteric sites will be more efficacious in interfering with for example protein-protein interactions, which notoriously have been difficult as drug targets. Allosteric agents will, for example have the possibility of stabilising a conformation of the biological target molecule where major parts of the protein-protein interface are vastly different from the one enabling the normal interaction.

Metal-ion site engineering in 7TM proteins – Metal-ions play a fundamental role in biology

In natural proteins they are involved in functional purposes such as electron transfer or

catalysis or in structural purposes stabilizing the three-dimensional structure of the protein. It is well known that also several integral membrane proteins include binding sites for metal ions. Regardless of whether the metal-ions play a functional or structural role, the specific properties exclusive to metals are utilized. In the proteins, the particular properties of the metal-ions may be fine-tuned by the amino acids defining the binding site to the application. An important general consideration is that metal-ions in fact offer the strongest binding interaction when viewed on a per atom basis compared to other ligands (I.D. Kuntz et al., Proc. Natl. Acad. Sci. USA, 96(18), 1999, pp. 9997-10002). The coordination of metal ions to metal ion binding sites is well characterized in numerous high-resolution X-ray and NMR structures of soluble proteins; for example, distances from the chelating atoms to the metal ion as well as the preferred conformation of the chelating side chains are known (e.g. J.P. Glusker, Adv. Protein Chem. 42, 1991, pp. 3-76; P. Chakrabarty, Protein Eng. 4, 1990, pp. 57-63; R. Jerigan et al., Curr. Opin. Struct. Biol. 4, 1994, pp. 256-263).

Thus, metal-ion binding in proteins is one of the most well characterised forms of receptor-ligand-protein interactions known. Hence, characterising a metal ion-binding site in a membrane protein using, for example, molecular models and site directed mutagenesis can yield information about the structure of the membrane protein and importantly where the "ligand" (metal ion) binds (e.g. Elling et al. Fold. Des. 2(4), 1997, pp. S76-80).

The forces that control metal-ion binding - Amino acid residues that function as effective metal binding residues are typically those that contain electron-donation atoms (S, O or N) (J.P. Glusker, Adv. Protein Chem., 42, 1991, pp. 1-76). Although this group includes amino acids such as Ser, Lys, Arg and Tyr, the strongest interactions typically involve Asp, Glu, Cys and His. Binding of a metal-ion to a ligand (a residue or an organic compound) can be considered in terms of Lewis acid base theory (J.P. Glusker, Adv. Protein Chem., 42, 1991, pp. 1-76). According to the Lewis concept, an acid is any species that can accept a pair of electrons, and a base is any species that can donate a pair of electrons. Consequently a metal acts as a Lewis acid when accepting an electron pair, and the ligand acts as a Lewis base when donating an electron pair. The nature of this electron transfer depends on the atoms involved, i.e. on the polarizability. On the basis of polarizability, metal ions may be classified as being hard/soft, hard meaning difficult to polarize. Important examples of cations such as Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+} and Ni^{2+} are classified as being 'borderline' (J.P. Glusker, Adv. Protein Chem., 42, 1991, pp. 1-76), i.e. neither hard nor soft. The softer the metal-ion is (more polarizability), the greater is the

tendency to form π bonds between the metal-ion and the ligand, whereas a hard acid binds hard bases by ionic forces.

Residues useful in metal-ion binding site engineering include for example aspartate, glutamate, histidine or cysteine. Aspartate and glutamate residues may carry one negative charge. Each oxygen atom has two lone pairs disposed at 120° to its C-O bond and in the plane of the carboxyl group. D.W. Christianson et al. (Am. Chem. Soc., 110(16), 1988, pp. 5560-5) showed that Zn(II) preferentially coordinates with the *syn* lonepair of the carboxylates (Z form) (Fig. 2). This was also observed by Carrell et al. (J. Am. Chem. Soc., 110(26), 1988, pp. 8651-6), which collected data from the Cambridge Structural Database. They showed that the syn-oriented lone pair of the carboxylate oxygen is preferred for cation binding. The metal-oxygen distance was in the range 2.3-2.6 Å and it is common to have 'direct bonding', in which the metal lies equidistant from the two oxygen atoms of the carboxylate group. In addition the metal-ion seems generally to lie in the plane of the carboxyl group. One carboxyl group can bind one or two metal ions, e.g. leucine aminopeptidase (Straeter et al., Biochemistry, 34(45), 1996, pp. pp. 14792-14800).

Histidine residues are involved in binding of metal-ions in a variety of enzymes and are the most abundant ligands in zinc binding sites (I. L. Alberts et al.,Protein Sci., 7(8), 1998, pp. 1700-1716). The imidazole ring exists in two tautomeric forms in which the proton is either on the Nε or Nδ nitrogen. The ratio is approximately 80% Nε-H and 20% Nδ-H (Fig. 3) (W.F. Reynolds et al., J. Am. Chem. Soc., 95(2), 1973, pp. 328-331). However, it is the Nδ-H form that predominantly binds the metal-ion (P. Chakrabarti, Protein Eng., 4(1), 1990, pp. 57-63). Steric factors may be responsible for reversing the preference of the two tautomeres when the histidyl is bound to a metal ion. Another factor may be due to the structural properties of histidyl, where the Nε-metal coordinated form may be stabilized by Nδ-backbone carbonyl hydrogen bonding to a preceding turn in an α-helix (P. Chakrabarti, Protein Eng., 4(1), 1990, pp. 57-63). Freeman (Inorganic Chemistry (G.L. Eichhorn, ed.), 1973, pp. 121-166) found that the metal-nitrogen bond of histidyl can be up to 30° from the imidazole plane and R. Candlen (J. Am. Chem. Soc., 110(26), 1967, pp. 8651-6) showed that the metal-Nε2-Cβ angles could range from 121° to 131° indicating a certain flexibility in the coordination, i.e. the metal-ion coordination does not have to be perfect for the metal to bind to the residue. The distance between the coordinated nitrogen and the metal is in the range 2.05-2.10 Å (I. L. Alberts et al.,Protein Sci., 7(8), 1998, pp. 1700-1716).

Coordination geometry - The most common structures of metal coordination spheres are octahedral, tetrahedral and square planar geometry's (F.A. Cotton et al, Basic Inorganic Chemistry (John Wiley & Sos, Inc.), 1995) (Fig. 4).

The coordination number and geometry depend on the nature of the metal and the size of the ligands (metal ion chelators, protein sidechains, water etc.), and is usually as high as possible. The surrounding ligand atoms arrange in a geometry that minimizes the repulsive energy between them (J.P. Glusker, Adv. Protein Chem., 42, 1991, pp. 1-76).

Zinc(II) (Zn(II)) and copper(II) (Cu(II)) are examples of two metal ions useful in metal-ion binding site engineering. They are both well-investigated transition metals. Zinc is involved in many hydrolytic enzymes, such as carboxypeptidase and carbonic anhydrase, which utilize zinc in the active site (L. Stryer, Annu. Rev. Biochem., 37, 1968, pp. 25-50). Copper is also found in a number structures, e.g. azurin (E.T. Adam et al., J. Mol. Biol., 123(1), 1978, pp. 35-47).

Zn(II) are most often found in a tetrahedral and octahedral geometry (D.W. Christianson, Adv. Protein Chem., 42, 1991, pp. 281-355). The mean zinc-ligand distance in protein coordination spheres is approximately 2 Å (2.05Å for histidine as a ligand) (P. Chakrabarti, Protein Eng., 4(1), 1990, pp. 57-63).

Cu(II) are most often found in a square planar or a distorted octahedral geometry (see Fig. 4) (J.P. Glusker, Adv. Protein Chem., 42, 1991, pp. 1-76). The coordination octahedron of Cu^{2+} is found not to contain six bonds of equal lengths, but has four short bonds (~2.0Å) and two long bonds (~2.4Å) (in trans) (Jahn and Teller, Proc. R. Soc. London, Ser. A 161, 1937, 220-235). Cu^{2+} usually bind its ligands (N & O) stronger than Zn^{2+} (Irving and Williams, Nature, 162, 1948, pp. 746) giving Cu^{2+} a different binding.

Engineering of new metal-ion binding sites - Due to the many favorable properties of metal-ions, engineering of new metal-ion binding sites in proteins have been performed in numerous cases although mostly in soluble proteins.

Several examples where metal-ion site engineering has been included in the design strategy of *de novo* proteins have been reported. A common obstacle in *de novo* design of a protein is that the fold of the protein often resembles a state in between a molten globule and a native folded protein presumably due to the lack of specific tertiary interactions. Engineering of metal-ion sites have been utilized to introduce positive interactions in the

protein fold stabilizing the folded native conformation as opposed to the molten globule state. A by now classic example of the elegant use of metal-ion site engineering was performed by Charlie Craik and coworkers in trypsin (J.N. Higaki, Biochemistry, 29, 1990, pp. 8582-8586). By introducing a histidine near the active site catalytic triad, a metal-ion switch was introduced. Upon metal-ion chelation, another histidine, that was normally part of the catalytic triad, would engage in metal-ion ligation with the introduced histidine and thereby render itself inaccessible for the normal catalytic function.

In one embodiment of the invention, metal-ion sites are introduced in 7TM receptors. Engineering of artificial metal ion binding sites into membrane proteins has been employed to explore the structure and function of these proteins. Thus, C.E. Elling et al., *Nature 374*, 1995, pp. 74-77, have reported how the binding site for a proto-type antagonist for the tachykinin NK-1 receptor could be converted into a metal ion-binding site by systematic substitution of residues in the binding pocket with His residues. Accordingly, a tridentate zinc-site was constructed, composed of two histidine residues located in an *I* and *I+4* position at the exterior end of TM-V (V:01 and V:05) and a single His residue located in TM-VI (VI:24).

If side chains of amino acid residues participating in metal ion binding are known, it imposes a distance constraint on the protein structure which can be used in the interpretation of unknown protein structures (C.E. Elling and T.W. Schwartz, *EMBO J. 15*(22), 1996, pp. 6213-6219; C.E. Elling et al., *Fold. Des. 2*(4), 1997, pp. S76-80).

Recently the generation of an activating metal-ion binding site has been reported for the β_2-adrenergic receptor, where the binding site for the normal catecholamine ligands was exchanged with a metal-ion site through specific substitutions in the binding pocket for the agonists (C.E. Elling et al, *PNAS 96*, 1999, pp. 12322-12327). This metal-ion binding site could be addressed also with metal-ions in complex with metal-ion chelators, i.e. small organic compounds binding metal-ions. The activating metal-ion site has successfully been transferred to another 7TM receptor, the tachykinin NK1 receptor (B. Holst et al., Mol. Pharmacol. 2000, 58: 263-270). However, none of the above-mentioned documents address the concept of using a chemical "anchor" in the drug discovery process.

Metal-ion binding sites are constructed by mutating one or more amino acid residue in the biological target molecule into residues, which can bind metal-ions. These are usually His, Cys, Asp, or Glu residues but could also be Trp, Tyr, Ser, Thr, Lys, Arg, Asn, Gln and Met. Additionally an engineered site may utilize electron donating groups from the polypeptide

backbone. It should be noted that also non-natural amino acids, which can bind metal-ions could be used, provided that a suitable method is employed to introduce these or a precursor for these - which can be chemically modified to become a metal-ion binding residue - into the biological target molecule. A metal-ion site includes one, two, three or four amino acid residues although more residues also can occur. In a specific embodiment of the invention the metal-ion binding site includes two or more residues, which allow for the metal-ion to bind also the metal-ion chelator. Often single metal-ion binding residue(s) are already present in the biological target molecule in a suitable location and consequently only a single extra metal-ion binding residue needs to be introduced in the spatial vicinity through mutational substitution of the residue found in the wild-type of the biological target molecule.

In order for two or more residues to make up a metal-ion binding site, the metal-ion binding atom of the amino acid residues need to be located or be able to move into a location which satisfies the geometrical criteria for making a metal-ion binding site with the particular metal-ion used, which will be known to the skilled person (I. Lalbert, 1998, Protein Science, 7: 1700-1716, B. Lvallee, 1990, Biochemistry, 29(24):5647-5659). Hellinga HW et al., J. Mol. Biol., 222(3), 1991, pp. 763-85). When engineering a metal-ion binding site in a 7TM receptor a general classification of the site may be performed based on the general placement of the introduced amino acid sidechains chelating the metal-ion. The metal-ion binding sites may be described as being either intra-helical, i.e. the residues composing the site are located on the same transmembrane helix; as being inter-helical, i.e. the residues composing the site are located on at least two transmembrane helices; or generally as involving residues within transmembrane segments and/or loops and turns of the protein. Whereas the residues defining an intra-helical metal-ion binding site are located on the same face of the helix, for example in an *i, i+4* manner, residues defining inter-helical residues are located on opposing faces of the involved helices and the site may be composed of one or more residues from each helix.

An important part of a drug discovery process is to increase the affinity of a test compound through the establishment of just a single or a few secondary chemical interaction(s) besides the anchoring binding of the metal-ion part of the complex. Thus, in order to facilitate the establishment of such a suitable secondary chemical interaction, the anchoring metal-ion sites are may be built into the biological target molecules at sites where knowledge from the known three-dimensional structure or from models of the three-dimensional structure indicate, that a suitable chemical moiety is present in the vicinity of the engineered metal-ion site. For example, if an acid residue, Asp or Glu, or a basic

residue, Arg, Lys or His, are found in a suspected exposed manner and in a supposed charged form in an certain epitope of a biological target molecule, a metal-ion site can be build in the vicinity of the charged residue, and chemical modifications of a suitable metal-ion chelate can be performed in order to establish e.g. a charge-charge interaction with the supposedly charged residue in the biological target molecule. In a similar manner, sites for engineering of metal-ion sites can be chosen in order to establish other types of suitable, secondary site chemical interactions through appropriate chemical modifications of the test compound. Secondary chemical interactions include the binding of second metal-ion between the test compound and the secondary site residue. It should be noted that chemically reactive groups with suitable chemical reactivity can be introduced into the test compound in order for these to selectively react with the intended, particular secondary site residue in the biological target molecule, and not with such residues in general in the biological target molecule, due to the close proximity in which the reactive group on the test compound is brought through the binding of the test compound to the metal-ion binding site in the biological target molecule.

However, many proteins contain natural metal-ion binding sites. These metal-ion sites serve either structural purposes, for example stabilizing the three-dimensional structure of the protein, or they serve functional purposes, where the metal-ion may for example be part of the active site of an enzyme. It is well known that also several integral membrane proteins include binding sites for metal ions. The coordination of metal ions to metal ion binding sites is well characterized in numerous high-resolution X-ray and NMR structures of soluble proteins; for example, distances from the chelating atoms to the metal ion as well as the preferred conformation of the chelating side chains are known (e.g. J.P. Glusker, *Adv. Protein Chem. 42*, 1991, pp. 3-76; P. Chakrabarty, *Protein Eng. 4*, 1990, pp. 57-63; R. Jerigan et al., *Curr. Opin. Struct. Biol. 4*, 1994, pp. 256-263). Thus, metal-ion binding in proteins is one of the most well characterised forms of ligand-protein interactions known. Hence, characterising a metal ion-binding site in a membrane protein using, for example, molecular models and site-directed mutagenesis can yield information about the structure of the membrane protein and importantly where the "ligand" (metal ion) binds (e.g. Elling et al. *Fold. Des.* 2(4), 1997, pp. S76-80).

Selection of a suitable metal-ion binding site in 7TMs

As discussed above, metal-ion binding sites in 7TMs may be found in the helices or in the loops. The binding site may be intrehelical, i.e. within the same helix or interhelical, i.e. involving two or more helices. In the following is given single positions in the different

transmembrane segments of a 7TM receptor; these single positions are positions which are suitable metal-ion binding sites:

Single Positions:

transmembrane segment-I:

[I:01]
[I:03]
[I:04]
[I:06]
[I:07]
[I:10]
[I:11]
[I:14]
[I:17]
[I:18]
[I:21]
[I:22]

or in positions

transmembrane segment-II:

[II:05]
[II:06]
[II:07]
[II:09]
[II:10]
[II:13]
[II:14]
[II:17]
[II:18]
[II:20]
[II:21]
[II:22]
[II:24]
[II:25]
[II:26]

or in positions

transmembrane segment-III:

[III:04]
[III:05]
[III:08]
[III:09]
[III:11]
[III:12]
[III:13]
[III:15]
[III:16]
[III:17]
[III:18]
[III:19]
[III:20]
[III:21]
[III:22]
[III:23]
[III:24]
[III:25]
[III:26]
[III:27]
[III:28]
[III:29]
[III:30]
[III:32]

or in positions

transmembrane segment-IV:

[IV:01]
[IV:02]
[IV:05]
[IV:06]
[IV:09]
[IV:10]
[IV:12]
[IV:13]
[IV:14]
[IV:16]
[IV:17]

[IV:18]
[IV:19]
[IV:20]

or in positions

transmembrane segment-V:
[V:01]
[V:04]
[V:05]
[V:08]
[V:09]
[V:12]
[V:13]
[V:16]
[V:17]
[V:20]
[V:21]
[V:23]
[V:24]
[V:27]
[V:28]

or in positions

transmembrane segment-VI:
[VI:-06]
[VI:-04]
[VI:-03]
[VI:-02]
[VI:-01]
[VI:01]
[VI:02]
[VI:03]
[VI:05]
[VI:06]
[VI:08]
[VI:09]
[VI:12]
[VI:13]
[VI:16]

[VI:17]
[VI:19]
[VI:20]
[VI:21]
[VI:23]
[VI:24]

or in positions

transmembrane segment-VII:
[VII:02]
[VII:03]
[VII:05]
[VII:06]
[VII:07]
[VII:08]
[VII:09]
[VII:10]
[VII:11]
[VII:12]
[VII:13]
[VII:15]
[VII:16]
[VII:17]
[VII:19]
[VII:20]

In the following is given preferred single positions in the different transmembrane segments of a 7TM; these single positions are preferred positions which are suitable metal-ion binding sites:

Preferably - Single

TM-I:
[I:03]
[I:07]
[I:10]
[I:14]

TM-II:

[II:17]
[II:18]
[II:20]
[II:21]
[II:24]
[II:25]

TM-III:

[III:04]
[III:05]
[III:08]
[III:09]
[III:19]
[III:23]
[III:27]
[III:29]
[III:32]

TM-IV:

[IV:12]
[IV:13]
[IV:16]
[IV:17]
[IV:19]
[IV:20]

TM-V:

[V:01]
[V:04]
[V:05]
[V:08]
[V:09]
[V:12]

TM-VI:

[VI:-01]

[VI:-02]
[VI:09]
[VI:16]
[VI:19]
[VI:20]
[VI:23]
[VI:24]

TM-VII:
[VII:02]
[VII:03]
[VII:06]
[VII:07]
[VII:10]
[VII:16]

In the following is given specific sites in the different transmembrane segments of a 7TM; these specific sites are sites which are suitable metal-ion binding sites:

transmembrane segment-I:
[I:03;I:07]
[I:06;I:10]
[I:10;I:14]
[I:14;I:18]
[I:17;I:21]
[I:18;I:22]

or in positions

transmembrane segment-II:
[II:05;II:09]
[II:06;II:10]
[II:09;II:13]
[II:17;II:21]
[II:20;II:24]
[II:21;II:25]

or in positions

transmembrane segment-III:

[III:04;III:08]
[III:05;III:09]
[III:08;III:12]
[III:09;III:13]
[III:11;III:15]
[III:12;III:16]
[III:15;III:19]
[III:19;III:23]
[III:20;III:24]
[III:21;III:25]
[III:22;III:26]
[III:23;III:27]
[III:24;III:28]

or in positions

transmembrane segment-IV:
[IV:01;IV:05]
[IV:02;IV:06]
[IV:05;IV:09]
[IV:06;IV:10]
[IV:12;IV:16]
[IV:13;IV:17]
[IV:14;IV:18]

or in positions

transmembrane segment-V:
[V:01;V:05]
[V:04;V:08]
[V:05;V:09]
[V:08;V:12]
[V:09;V:13]
[V:12;V:16]
[V:13;V:17]
[V:16;V:20]
[V:17;V:21]
[V:20;V:24]
[V:23;V:27]
[V:24;V:28]

or in positions

transmembrane segment-VI:
[VI:-04;VI:01]
[VI:-03;VI:02]
[VI:-02;VI:03]
[VI:01;VI:05]
[VI:02;VI:06]
[VI:05;VI:09]
[VI:08;VI:12]
[VI:09;VI:13]
[VI:13;VI:17]
[VI:16;VI:20]
[VI:17;VI:21]
[VI:19;VI:23]
[VI:20;VI:24]

or in positions

transmembrane segment-VII:
[VII:02;VII:06]
[VII:03;VII:07]
[VII:06;VII:10]
[VII:07;VII:11]
[VII:09;VII:13]
[VII:12;VII:16]
[VII:13;VII:17]
[VII:15;VII:19]
[VII:16;VII:20]

or in positions

transmembrane segment-I and -II
[I:01;II:26]
[I:01;II:25]
[I:01;II:22]
[I:03;II:25]
[I:04;II:26]
[I:04;II:25]
[I:04;II:22]
[I:07;II:25]
[I:07;II:22]
[I:07;II:21]

[I:07;II:18]
[I:10;II:18]
[I:10;II:14]
[I:10;II:13]
[I:11;II:18]
[I:11;II:14]
[I:14;II:18]
[I:14;II:13]
[I:14;II:14]
[I:14;II:10]
[I:18;II:10]
[I:18;II:07]

or in positions

transmembrane segment-I and -VII
[I:03;VII:07]
[I:03;VII:03]
[I:07;VII:07]

or in positions

transmembrane segment-II and -III
[II:20;III:04]
[II:24;III:04]

or in positions

transmembrane segment-II and -VII
[II:17;VII:10]
[II:18;VII:10]
[II:21;VII:07]

or in positions

transmembrane segment-III and -IV
[III:05;IV:17]
[III:05;IV:20]
[III:09;IV:16]
[III:09;IV:17]

or in positions

transmembrane segment-III and –V
[III:05;V:01]
[III:05;V:04]

[III:05;V:08]
[III:09;V:01]
[III:09;V:04]
[III:09;V:08]
[III:12;V:08]
[III:13;V:08]
[III:13;V:12]
[III:16;V:12]
[III:16;V:16]
[III:17;V:16]
[III:19;V:16]
[III:20;V:16]
[III:23;V:27]
[III:23;V:23]
[III:26;V:20]
[III:26;V:24]
[III:26;V:27]
[III:27;V:23]
[III:27;V:27]
[III:30;V:23]
[III:30;V:27]

or in positions

transmembrane segment-III and -VI

[III:05;VI:16]
[III:08;VI:16]
[III:08;VI:13]
[III:09;VI:13]
[III:12;VI:13]
[III:13;VI:09]
[III:16;VI:09]
[III:19;VI:09]
[III:19;VI:06]
[III:19;VI:05]
[III:19;VI:09]
[III:22;VI:05]
[III:23;VI:09]

[III:25;VI:02]
[III:25;VI:-03]
[III:26;VI:02]
[III:26;VI:-03]
[III:26;VI:-02]
[III:26;VI:-06]
[III:27;VI:-01]
[III:27;VI:-02]
[III:29;VI:-02]
[III:30;VI:-06]
[III:30;VI:-02]
[III:32;VI:-02]

or in positions

transmembrane segment-III and -VII
[III:04;VII:07]
[III:05;VII:06]
[III:09;VII:09]
[III:08;VII:06]
[III:08;VII:10]
[III:11;VII:10]
[III:12;VII:06]
[III:12;VII:10]
[III:12;VII:12]
[III:15;VII:16]
[III:19;VII:16]

or in positions

transmembrane segment-IV and -V
[IV;19;V:04]
[IV;20;V:04]
[IV;12;V:12]
[IV;16;V:08]

or in positions

transmembrane segment-V and -VI
[V:01;VI:20]
[V:01;VI:24]

[V:05;VI:20]
[V:05;VI:24]
[V:09;VI:20]
[V:09;VI:21]
[V:09;VI:17]

or in positions

transmembrane segment-VI and -VII
[VI:23;VII:02]
[VI:23;VII:05]
[VI:20;VII:02]
[VI:19;VII:05]
[VI:19;VII:02]
[VI:16;VII:02]
[VI:16;VII:06]
[VI:16;VII:09]
[VI:16;VII:10]
[VI:12;VII:08]
[VI:12;VII:09]
[VI:09;VII:12]
[VI:09;VII:15]
[VI:05;VII:16]
[VI:05;VII:15]

In the following is given preferred specific sites in the different transmembrane segments of a 7TM; these specific sites are preferred sites which are suitable metal-ion binding sites:

Preferably - *Intra*helical

TM-I:
[I:03;I:07]
[I:10;I:14]

TM-II:
[II:17;II:21]
[II:20;II:24]
[II:21;II:25]

TM-III:

[III:04;III:08]

[III:05;III:09]

TM-IV:

[IV:12;IV:16]

[IV:13;IV:17]

TM-V:

[V:01;V:05]

[V:04;V:08]

[V:05;V:09]

TM-VI:

[VI:16;VI:20]

[VI:19;VI:23]

[VI:20;VI:24]

TM-VII:

[VII:02;VII:06]

[VII:03;VII:07]

[VII:06;VII:10]

Preferably - *Inter*helical Sites

TM-I / TM-VII

[I:03;VII:07]

[I:07;VII:07]

[I:03;VII:03]

TM-II / TM-III

[II:20;III:04]

[II:24;III:04]

TM-II / TM-VII

[II:17;VII:10]
[II:18;VII:10]
[II:21;VII:07]

TM-III / TM-IV
[III:05;IV:17]
[III:05;IV:20]
[III:09;IV:16]
[III:09;IV:17]

TM-III / TM-V
[III:05;V:01]

TM-III / TM-VI
[III:08;VI:16]
[III:19;VI:09]
[III:23;VI:09]
[III:27;VI:-01]
[III:27;VI:-02]
[III:29;VI:-02]
[III:32;VI:-02]

TM-III / TM-VII
[III:08;VII:06]
[III:19;VII:16]

TM-IV / TM-V
[IV;19;V:04]
[IV;20;V:04]
[IV;12;V:12]
[IV;16;V:08]

TM-V / TM-VI
[V:01;VI:20]
[V:01;VI:24]
[V:05;VI:20]
[V:05;VI:24]

TM-VI / TM-VII

[VI:23;VII:02]

[VI:23;VII:06]

[VI:19;VII:02]

[VI:19;VII:06]

Selection of a suitable test compound (metal ion chelate) for use in a drug discovery process

The aim with a drug discovery process is to identify novel drug compounds with properties suitable for use in therapy or for diagnostic and/or prophylactic purposes.

From the description given above relating to the importance of identifying, optimising and selecting suitable test compounds it is evident that design of suitable libraries of such compounds is an important and necessary tool in order to enable a more efficient way of testing large numbers of test compounds and thus, in a much more easy and time saving manner collecting relevant information on how the test compounds should be modified in order to e.g. more strongly interact with the metal ion binding site or with residues in the vicinity of the metal ion binding site. In the following various libraries of test compounds are described and the libraries have been designed especially for the use in drug discovery processes.

In general, complexes of interest are chelates consisting of three major parts: the functionalised chelator, the metal ion *(central metal or coordinated metal)*, and displaceable ligands bound to the metal. When the metal chelate binds to the biological target the displaceable ligands are exchanged with groups from the biological target.

Functional groups in a ligand attached to the metal ion are the ligand's coordinating groups. A ligand attached through only one coordinating atom (or using only one coordination site on the metal) is called a monodentate ligand. A ligand that may be attached through more than one atom is multidentate, the number of actual coordinating sites being indicated by the terms bidentate, tridentate, tetradentate and so forth. Multidentate ligands attached to a central metal by more than one coordinating atom are called chelating ligands, or chelators. A chemical compound for use in the present context is at least bidentate, i.e. it is a so-called metal ion chelator.

Optimising the binding to the metal ion binding site of the biological target molecule containing a metal ion binding site

Primary interaction, the metal complex.

The nature of the complex can be altered depending on the metal ion binding site in the target protein. It is usually advantageous in the described drug discovery process to have labile complexes. This means that the complex formation is reversible with a relatively fast interchange of ligands, and the metal ion prefers the complexed structure due to a thermodynamic favorable situation.

The types of ligands can roughly be divided into three groups; (i) pure σ-donating ligands, e.g. amines or thiols, (ii) ligands with additional π-back bonding to an electron deficient π systems, e.g. pyridines, and (iii) ligands with additional π-back bonding to an electron rich π systems, e.g. thiophenes.

To enforce a low electron density at the metal center, use of ligands with back bonding capabilities is favorable, and this is further improved with electron deficient π systems. Firstly, back bonding strengthens the binding between the chelator and the metal, and secondly, removing electron density from the metal allows the metal site in the target to form stronger interactions with the metal.

Based on these considerations, chelators containing e.g. pyridine are of interest, but also other heteroaromatic systems, like imidazoles, pyrazoles, triazoles, oxazoles, thiazoles, quinolines etc. should be considered. In addition, phenolic and thiophenolic functionalities are known to act as chelating groups. Thus, possible chelators advantageous for the task are 2,2'-bipyridine, 8-hydroxyquinoline, 8-mercaptoquinoline, and 2-(2-pyridyl)thiophenol.

Secondly, there is the choice of metal ion. Both zinc and copper has proven useful for the present purpose, but other metal ions similar to these are applicable. However, for certain targets it may be advantageous to use a different metal to avoid endogenous metal sites or metal species. Other suitable metal ions may include, but is not limited to, cobalt, nickel, iron, manganese, as well as the heavier metals, like technetium, ruthenium, rhodium, palladium, osmium, platinum etc. The various metals have different electronic properties, thus a different metal will show different preferences for various electronic environments, both in the metal ion site in the target, as well as from the chelator.

Finally, the interaction between the metal and the metal site in the protein target can be modulated. Both histidine, with its imidazole moiety, and cysteine, with its thiol group, has proven successful as ligands to the metal. Cysteine is a pure σ-donor, while histidine also includes additional π-back bonding. Other residues that may be used in the metal sites are glutamates and aspartates.

As described above, the affinity between the metal, metal site and chelator is dependent on their individual properties. For some cases, a specific combination may turn out to be too weak for the purpose. For membrane-bound protein targets in general, the combination of 2,2'-bipyridine-based chelators and zinc seems to form a too weak complex to be useful, i.e. equilibrium constant log K ~ 6, when the assay requires low metal ion concentrations (< 1 μM). However, changing the metal to e.g. copper in combination with 2,2'-bipyridine seems to result in a complex of relevant strength, i.e. equilibrium constant log K ≥8. Similarly, by using e.g. 8-mercaptoquinoline as a chelator for zinc, the resulting complex is of relevant strength, i.e. equilibrium constant log K ≥8. These considerations may be redundant when high metal ion concentrations could be utilised in the assay conditions.

Optimising secondary interactions

When the metal complex bridging the biological target molecule and the chelator has been established, secondary interactions between the chelator and other residues in the target should be introduced. These are obtained by functionalising the chelators with proper functional groups at proper distances from the metal centre, targeting functionality in spatial proximity of the metal site.

This is achieved e.g. by testing a diverse library containing the metal ion chelating scaffold optimised for primary interactions and varying the functionalities at different distances from the coordination centre for achieving improved secondary interactions. Such a chemical optimisation can also be performed in a more targeted fashion using the structural information of the biological target molecules.

Identification of chemical interactions

The chemical optimisation of the test compound can be guided by detailed knowledge of the 3D structure(s) of the biological target molecule, preferentially determined in complex initially with the un-substituted metal-ion chelator and subsequently in complex with the

chemically modified metal-ion chelator in which attempts have been made to establish first one secondary interaction and subsequently further secondary or tertiary interactions. For some biological target molecules such as soluble proteins this can be achieved through for example crystallization and standard X-ray analysis procedures or through, for example NMR analysis of the complex in solution again using standard procedures.

For membrane proteins high resolution structures are in general not available. However determination of chemical interactions may be performed using a generic three-dimensional model of the membrane protein showing the spatial arrangement of the amino acid residues defining the area of the metal-ion binding site. Such a determination is then performed using site-directed mutagenesis of a least one amino acid residue potentially involved in interaction with said functional group of the test compound other than the metal ion. Followed by expression of the mutated membrane protein in a suitable cell, contacting said cell or a portion thereof including the mutated membrane protein with the test compound, and determining any effect on binding in a competitive binding assay using a labelled ligand of the membrane protein, detection of any changes in signal transduction from the membrane protein or using a chelating agent which is in itself detectable or labelled with a detectable labelling agent. If an amino acid residue involved in interaction with such a functional group of the test compound is mutated to one, which is not - this may be detected as a decrease in binding or other activity

Generation of new specific interactions

During the chemical optimisation of the test compound methods developed for structure-based drug discovery In general can be utilized, as knowledge of the 3D structure of the target epitopes makes it possible to apply classical structure-based approaches such as structure-based library design for the establishment of secondary and tertiary interaction sites for the lead compound In the target molecule. However, it should be noted, that a major advantage and difference of the present method is, that the lead compound is anchored to a particular site and thereby to a certain degree in a particular conformation in the biological target molecule through binding to the bridging metal-ion site while the compound is being optimised for chemical recognition with the target molecule.

In the case of membrane proteins suitable X-ray structures are generally not available. However, the molecular models are often rather detailed and in the case of the 7TM receptors they are in fact rather precise and correspond well with the X-ray structure of rhodopsin, which was recently published. Thus the combination of relatively good

molecular models (which have allowed for the construction of interhelical metal-ion sites) and the present method does to a certain degree compensate for the lack of detailed knowledge of the 3D structure of the target molecule because the lead compound is anchored and thereby create a fix-point for the subsequent medicinal chemical optimisation point guided by the molecular models.

By using relatively flexible spacers in between the metal-ion chelating moiety and the variable chemical moiety of the test compound it becomes possible to probe for interaction or binding to structurally and functionally interesting epitopes of the biological target molecule with chemical moieties, which due to their intrinsic low affinity would normally not be detectable in the analytical systems on their own. Due to the local high concentration of the chemical moieties, which is created by the tethering to the metal-ion chelating moiety bound to the metal-ion site, these compounds can now be detected.

Once detected, the interactions probed by flexible spacer arms can be improved by introduction of rigidity in the spacer group. Thus, presence of e.g. chain branching, higher-order bonds or ring systems in the spacer may enhance the affinity of the molecules. This step may be done for each separate secondary interaction, or it may be done in parallel for several functionalities.

The bonds formed may be of, but not limited to, one of the following types:
Ionic interactions, ion-dipole interactions, dipole-dipole interactions, hydrogen bond interactions, hydrophobic interactions, pi-stacking interactions, edge-on aromatic interactions, dispersion and inductions forces or metal complex interactions. More specifically:

1. charge-charge interaction (introduction of charged groups such as ammonium, phosphonium or sulphonium groups, or ionisable groups such as amino or carboxy groups).
2. hydrogen bond interactions (introduction of e.g. hydroxy, amino, keto amide, sulphonyl or other groups capable of interacting via formation of a hydrogen bond).
3. aromatic-aromatic interactions (introduction of an aromatic group in the test compound).
4. cation-π interactions (introduction of cationic groups such as ammonium groups or introduction of an aromatic moiety)
5. hydrophobic interactions (introduction of hydrophobic groups).

6. a second metal-ion bridge or anchor established through a metal-ion binding moiety on the variable part of the compound and a metal-ion binding residue on the biological target molecule.

The functionality can be introduced in a random fashion, or in a more targeted manner, taking advantage of known structural elements in the protein target.
When one secondary interaction has been established, introduction of further functional groups on the chelator will lead to further increase in binding affinity, and this process will be repeated until a satisfactory affinity is obtained. Alternatively, two or more functional groups capable of interacting with the protein may be introduced simultaneously.

The reactive groups used for forming secondary interactions should be compatible with the experimental set-up. Preferably, the functional groups selected should be compatible with properties required in a potential drug candidate.

Optimising the binding to the wild type biological target molecule

Exchange of metal-ion anchor with "ordinary" chemical interaction

In the case, where the initial binding of the metal-ion chelator was obtained through mutational introduction of an anchoring metal-ion site in the biological target molecule, a final step of optimization may have to be performed to obtain high affinity binding or potency on the wild-type target molecule without the metal-ion bridge. Through the approached described above, the metal-ion chelator lead compound will gradually be optimized for interactions with chemical groups in the biological target molecule spatially surrounding the metal-ion site - i.e. interactions with chemical groups found also in the wild-type target molecule. Thus, the test compound will gradually increase its affinity not only for the metal-ion site engineered molecule but also for the wild-type biological target molecule. When two to three secondary interaction points have been established, the affinity of the test compound for the wild-type target molecule, which is being tested in parallel with the metal-ion site engineered molecule, will have reached micro-molar affinities, i.e. a lead compound on the wild-type target molecule has been created. At this point one or more of the following three approaches will be followed:

1) structure-based further chemical optimisation of the compound in general aiming at improving recognition at various known chemical moieties of the target molecule;
2) structure-based further chemical optimisation of the compound at which the "metal-ion site bridge" is exchanged by a more classical type of chemical interaction with

the residue(s) which had been modified to create the metal-ion site in the biological target molecule. Here advantage can be taken of the fact that the geometry of the metal-ion site anchor is well known in general and, that relatively limited structure-based libraries can be established to create a new type of interaction;

3) further chemical optimisation of the compound through more-or-less random generation of chemical diversity in general in the compound.

Beyond the metal complex, optimising for the wild type biological target molecule

When an acceptable affinity of a functionalised chelator has been obtained when tested on the wild type biological target molecule, without the metal ion binding site, the test compound will be optimised for interactions with the wild type biological target molecule, i.e. the part involved in the complexation may be transformed into structural elements having interaction with residues in the wild type biological target molecule.

This optimisation may be done randomly, or in a targeted fashion, taking advantage of knowledge of the target.

Targets containing natural metal sites

A further aspect of the invention is to utilise naturally occurring binding sites, i.e. binding sites already present in the biological target. In this case, depending on the physiological surroundings, retaining the chelator may be advantageously for binding, while utilising physiological concentrations of endogenously occurring metal ions.

Libraries of chemical compounds

A library according to the invention contains three or more test compounds. Typically, a library based on structural information from biological target molecules contains from about 10 to about 100 compounds and typically there are from about 100 to about 10,000 compounds in more diverse libraries suitable for probing the vicinity of the metal ion binding site for secondary interactions in the biological target molecule.

Besides the chemical structure, the test compounds contained in the libraries normally fulfill certain criteria with respect to molecular weight (at the most 3000 such as, e.g., at the most 2000, at the most 1500, at the most 1000, at the most 750, at the most 500), number of hydrogen bond donors (at the most 15 such as, e.g. at the most 13, 12, 11, 10,

8, 7, 6 or at the most 5) and number of hydrogen bond acceptors (at the most 15 such as, e.g. at the most 13, 12, 11, 10, 8, 7, 6 or at the most 5). However, there may be cases where the molecular weight, number of hydrogen bond donors and/or number of hydrogen bond acceptors of a test compound of a library of the invention have other values than the above-mentioned.

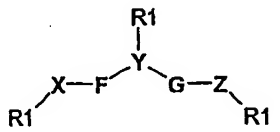
Chemical compounds, which are suitable for use in drug discovery processes involving biological target molecules having a metal-ion site, are any compounds that are capable of forming a complex with a metal ion.

More specifically, a chemical compound for use in a library according to the invention has at least two heteroatoms, similar or different, selected from the group consisting of nitrogen (N), oxygen (O), sulphur (S), selenium (Se) and phosphorous (P).

Chemical compounds, which have been found to be useful in libraries of the present invention, are typically compounds comprising a heteroalkyl, heteroalkenyl, heteroalkynyl moiety or a heterocyclyl moiety for chelating the metal ion.

The term "heteroalkyl" is understood to indicate a branched or straight-chain chemical entity of 1-15 carbon atoms containing at least one heteroatom. The term "heteroalkenyl" is intended to indicate a branched or straight-chain chemical entity of 2-15 carbon atoms containing at least one double bond and at least one heteroatom. The term "heteroalkynyl" is intended to indicate a branched or straight-chain chemical entity of 2-15 carbon atoms containing at least one triple bond and at least one heteroatom. The term "heterocyclyl" is intended to indicate a cyclic unsaturated (heteroalkenyl), aromatic ("heteroaryl") or saturated ("heterocycloalkyl") group comprising at least one heteroatom. Preferred "heterocyclyl" groups comprise 5- or 6-membered rings with 1-4 heteroatoms or fused 5- or 6-membered rings comprising 1-4 heteroatoms. The heteroatom is typically N, O, S, Se or P, normally N, O or S. The heteroatom is either an integrated part of the cyclic, branched or straight-chain chemical entity or it may be present as a substituent on the chemical entity such as, e.g., a thiophenol, phenol, hydroxyl, thiol, amine, carboxy, etc. Examples of heteroaryl groups are indolyl, dihydroindolyl, furanyl, benzofuranyl, pyridyl, pyrimidyl, pyrazoyl, benzothiazoyl, quinolinyl, triazolyl, imidazolyl, thiazolyl, tetrazolyl and benzimidazolyl. The heterocyclyl group generally includes 2-20 carbon atoms, and 1-4 heteroatoms.

Particularly useful chemical compounds in libraries of the present invention are those having at least two heteroatoms connected according to the general formula I abbreviated as Che-R1



Formula I

wherein F is N, O, S, Se or P; and G is N, O, S, Se or P;

X, Y and Z, which are the same or different, are straight or branched C_1-C_{12} alkyl, C_1-C_{12} alkenyl, C_1-C_{12} alkynyl, C_1-C_{12} cyclyl, aryl, C_1-C_{12} heteroalkyl, C_1-C_{12} heteroalkenyl, C_1-C_{12} heteroalkynyl, C_1-C_{12} heterocyclyl, heteroaryl;

R1 may be present anywhere on the X, Y and/or Z moiety and it may be present on X, Y and/or Z up to as many times as possible, i.e. if X is –CH2-CH2-, then R1 may be present on the first and/or second carbon atom one or several times; R1 could optionally be hydrogen;

X may together with Y and/or Z fuse to form a cyclic ring system;
Y may together with X and/or Z fuse to form a cyclic ring system;
X, Y and Z may together fuse to form a cyclic ring system;

R^1 corresponds to a structure –A-B-C, wherein the element A is a coupling or connecting moiety, B is a spacer moiety and C is a functional group; –B- may be substituted one or more times with a further C, which may be the same or different, and

A linked to be –A-B-C is selected from the group consisting of:

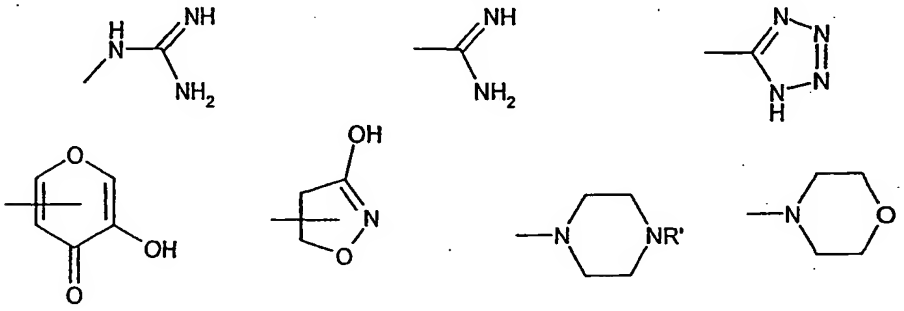
-O-, -S-, -NH-, -N=, -N<, -CH_2-, -C(=O)-, -PO_3-, -PO_2NH-, -$NHPO_2$, -NHP(O)<, -C≡C-, -CH=CH-, -SO-, -SO_2-, -COO-, -CONR"-, -NR'CO-, -NR'SO_2-, -SO_2NR"-, -CH(OH)-, -CR'(OH)-, -CR'(O-alk)-, -N-alk-, aryl, cycloalkyl, heteroaryl, heterocycloalkyl etc., and the term "alk" includes straight or branched alkyl, straight or branched alkenyl and straight or branched alkynyl; R' is H or lower alk, i.e. C_1-C_6; R" is as defined below;

-B- is absent or selected from the group consisting of:

H, alkyl, straight or branched alkyl, alkenyl (straight or branched), alkylnyl (straight or branched), aryl, cycloalkyl, heteroaryl, heterocycloalkyl, alkyloxyalkyl, alkylaminoalkyl,

-C is absent or selected from the group consisting of:

-H, -OH, -NR"R"', -CONR"R"', -COOR", -OCOR", -COR", $-SO_2NR"R"'$, -SH, -S-S-alk, -NHCOR", -NR"COR"', $NHSO_2R"$, $-NHCONH_2$, -NH-CN, -F, -Cl, -Br, -I; $-SCF_3$, $-CF_3$, $-OCF_3$, $-SCH_3$, -SR", -CN, $-N(CN)_2$,$-NO_2$, $-OCH_3$, -OR', $-NH_2$, -NHAlk, -NHMe, $-NHAlk_2$, $-NMe_2$, -NMeAlk, $-N(Alk)_3^+$, heteroaryl, heterocycloalkyl



and R" and/or R"' has the same meaning as given for B above optionally substituted with one or more C;

in those cases where a compound has two or more R^1 in positions adjacent to each other the –A- and/or –B- elements from the two individual R^1 may form a cyclic ring system;

in those cases where B is absent R^1 is –A-C or –A and in those cases where C is absent R^1 is –A-B or –A;

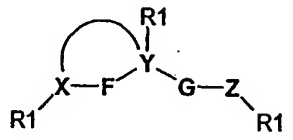
in some cases, A may be absent and then $-R^1$ is –B-C or –C, and B may be substituted one or more times with C, which may be the same or different;

the total number of atoms (X+F+Y+G+Z) excluding hydrogen atoms is at the most 25;

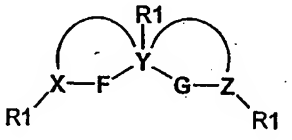
the total number of heteroatoms in (X+F+Y+G+Z) is at the most 6; and

the size of a ring is at the most 14 atoms, preferably 5 or 6 atoms.

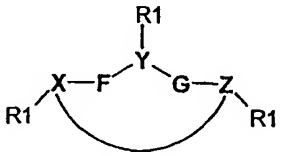
As mentioned above X, Y and/or Z may fuse to form one or more rings. Thus, X-F-Y may be part of a heterocyclyl ring system:



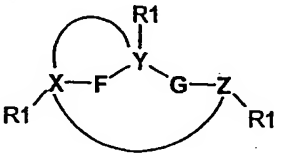
Alternatively, X-F-Y and Y-G-Z may be part of heterocyclyl ring systems:



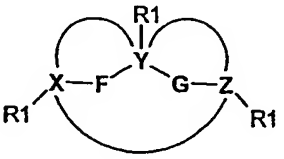
X-F-Y-G-Z may also be part of heterocyclyl ring systems:



X-F-Y and X-F-Y-G-Z may be part of heterocyclyl ring systems:



Furthermore, X-F-Y and Y-G-Z and X-F-Y-G-Z may be part of heterocyclyl ring systems:



In the present context, the term "alkyl" is intended to indicate a branched or straight-chain, saturated chemical group containing 1-15 such as, e.g. 1–12, 1-10, preferably 1-8, in particular 1-6 carbon atoms, such as methyl, ethyl, propyl, isopropyl, butyl, sec. butyl, tert. butyl, pentyl, isopentyl, hexyl, isohexyl, heptyl etc.

The term "alkenyl" is intended to indicate an unsaturated alkyl group having one or more double bonds.

The term "alkynyl" is intended to indicate an unsaturated alkyl group having one or more triple bonds.

The term "cycloalkyl" is intended to denote a cyclic, saturated alkyl group of 3-7 carbon atoms.

The term "cycloalkenyl" is intended to denote a cyclic, unsaturated alkyl group of 5-7 carbon atoms having one or more double bonds.

The term "aryl" is intended to denote an aromatic (unsaturated), typically 6-membered, ring, which may be a single ring (e.g. phenyl) or fused with other 5- or 6-membered rings (e.g. naphthyl or anthracyl).

The term "alkoxy" is intended to indicate the group alkyl-O-.

The term "amino" is intended to indicate the group –NR"R"' where R" and R"' which are the same or different, have the same meaning as R* in formula I. In a primary amine group, both R" and R"' are hydrogen, whereas in a secondary amino group, either but not both R" and R"' is hydrogen. In a tertiary amino group neither of R" and R"' is hydrogen. R" and R"' may also be fused to form a ring.

The term "ester" is intended to indicate the group COO-R", where R" is as indicated above except hydrogen, -OCOR*, or a sulfonic acid ester or a phosphonic acid ester.

In the formula I above it is contemplated that if the valency of the heteroatoms F and/or G is more than 2 then further X, Y and/or Z groups may be present adjacent to the F and/or G groups.

Specific libraries

A library according to the invention may contain chemical compounds having a specific characteristic feature in common or it may contain chemical compounds representing a broad diversity of chemical functional groups and/or chemical structures. The chemical compounds of a library may also have a basic common structural element such as, e.g., 2.2'-bipyridine.

For simplicity, the Formula I and specific subgroups of the general structure are denoted here simply as Che-R^1 or more specifically Che-A-B-C, wherein Che constitutes the different chelating scaffolds optionally substituted further with one or more, the same or different, R^1 or more specifically A-B-C groups.

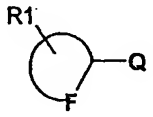
Accordingly, a library of the present invention may, e.g., contain

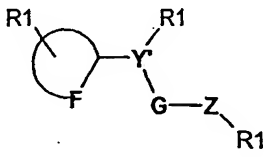
- Test compounds having the same chemical functional group, C.
- Test compounds having the same or almost same spacer moiety, B, in order to establish a distance from the heteroatom containing skeleton to the chemical functional group; in such a library, the chemical functional group may be the same or different.
- Test compounds which in principle are prepared by the same method and/or which have the same kind of attachment, A, to the basic common structural element.
- Test compounds which are capable of establishing a non-covalent interaction.
- Different test compounds which are chelated with the same metal ion.
- The same test compound, which is chelated with different metal ions.

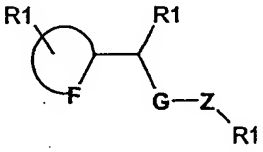
Libraries containing chemical compounds of the following general formulas are of specific interest in the present context. The following formulas are based on the formula I above and F and/or G have the same meaning as indicated above, i.e. F and/or G are heteroatoms. Q is a structural element containing a heteroatom. A circle indicates a cyclic alkyl, alkenyl, aryl, heteroalkyl, heteroalkenyl, heteroalkynyl or heteroaryl ring having from 3-7 atoms in the ring. R^1 has the same meaning as indicated above and, when more than one R^1 is present they may be the same or different. If no specific position is given for the radical, the radical may be placed anywhere in the cyclic system and there may also be as many radicals as there is positions possible in the structure. Other symbols employed in the formulas below have the same meaning as given under formula I above. In the

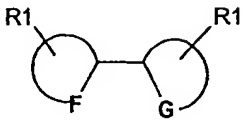
formulas below, the structure of the compounds are given in different structure levels. First in a very general form and then in more and more specific forms.

More specifically, a library according to the present invention comprises test compounds, which have one of the following structures. Y' is the remainder of the group Y which also includes Y' being absent, i.e. G being directly linked to the ring. The coordinating atom F is included in a 5- or 6-membered aromatic, unsaturated or saturated heterocycle containing between one and three heteroatoms and the coordinating atom G is either included in a 5- or 6-membered aromatic, unsaturated or saturated ring or an open chain. Preferably, F is N, O or S; and G is N, O or S:

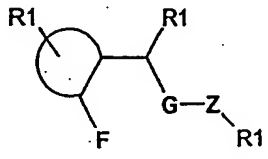


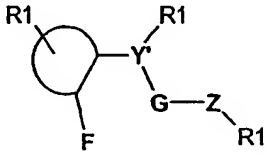


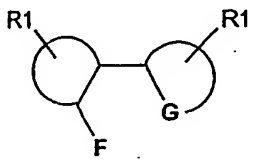




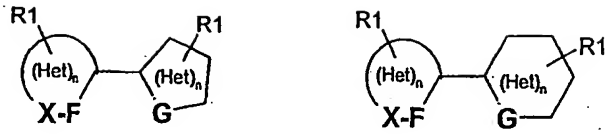
In the following subclass the coordinating atom F is appended to an aromatic, unsaturated or saturated 5- or 6-memebered ring. Preferably, F is N, O or S; and G is N, O or S.



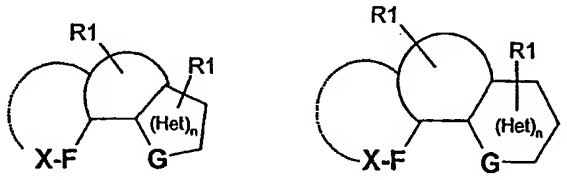




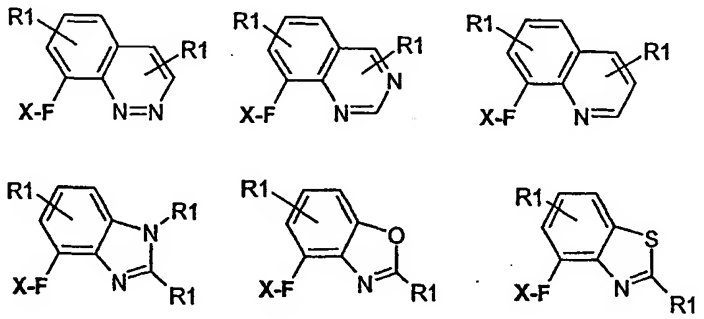
In the following biheterocyclyl subclass the coordinating atom G is included in a 5-or 6-membered aromatic, unsaturated or saturated heterocycle containing between one and four heteroatoms and the coordinating atom F contained within an aromatic, unsaturated or saturated 5- or 6-memebered heterocycle containing between one and four heteroatoms. Preferably, F is N, O or S; and G is N, O or S.



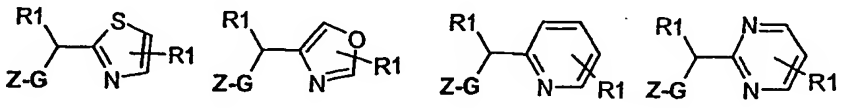
In the following subclass the coordinating atom G is included in a 5- or 6-membered aromatic, unsaturated or saturated heterocycle containing between one and three heteroatoms and the coordinating atom F appended to an annelated aromatic, unsaturated or saturated 5- or 6-memebered ring. X-F can optionally be included in a fused ring as indicated by the dashed line. Preferably, F is N, O or S; and G is N, O or S.



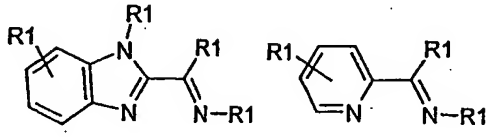
The annelated derivatives may be substituted with one or more R^1 moieties. Thus, a library of the present invention may contain mono-, di-, tri-, tetra-, pentasubstituted derivatives.



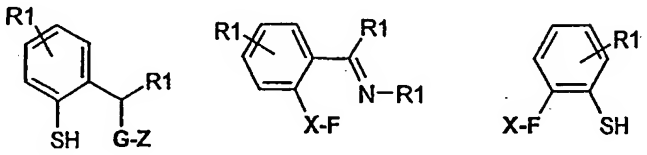
Suitable heterocyclic coordinating rings could be appended with coordinating moieties G to produce other chelating scaffolds containing one or more R1 groups.



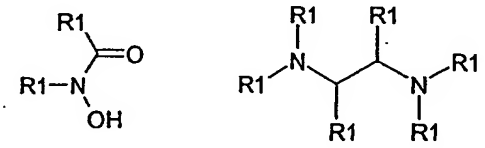
Typical coordinating sacffolds of this type are imine moieties appended to coordinating heterocycles.



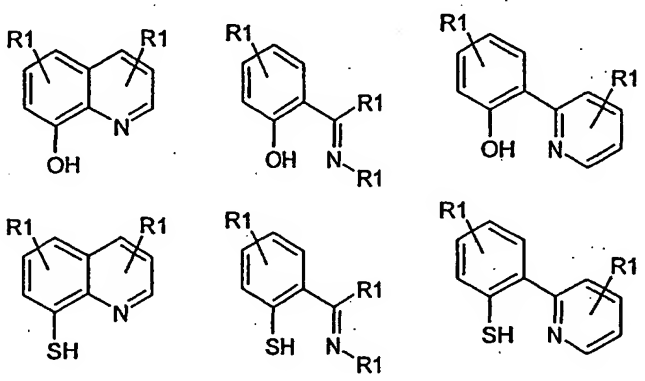
Alternatively, the coordinating groups, e.g. thiol and imine, may be attached to a ring moiety containing one or more R1 groups.



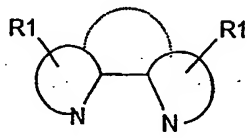
Other suitable open-chain chelating scaffolds are hydroxamic acids or 1,2-diamine coordinating moieties containing one or more R1 groups.



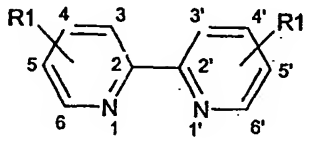
Chelator scaffolds containing one or more R1 groups of particular value are:



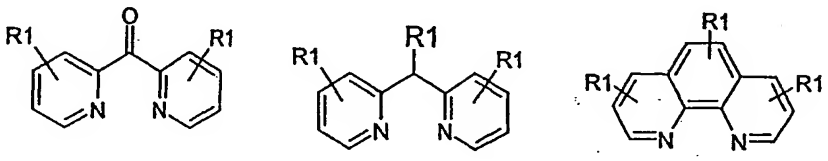
Useful nitrogen containing biheterocyclyl chelator scaffolds of particular interest are:



and, especially, pyridine containing systems of the following type

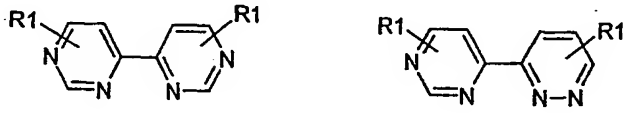


Other useful pyridyl-containg systems are systems such as

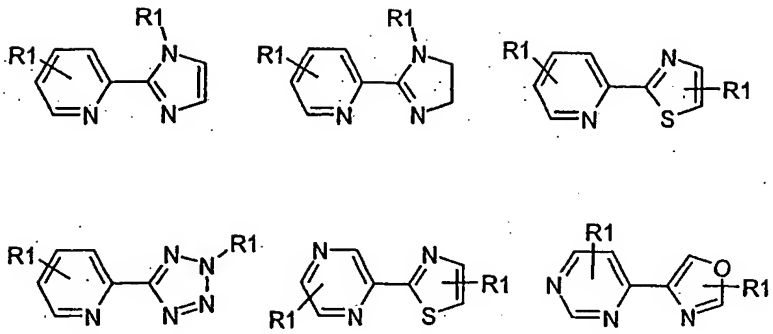


2-pyridyl systems may also be connected to other six-membered nitrogen containing rings having one nitrogen adjacent to the connecting bond, such as

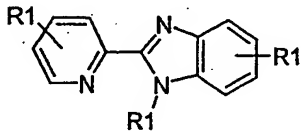
Non-pyridyl six-membered nitrogen containing aromatic rings may also be coupled to another non-pyridyl six-membered nitrogen containing ring where both ring systems having one nitrogen adjacent to the connecting bond, form useful scaffolds



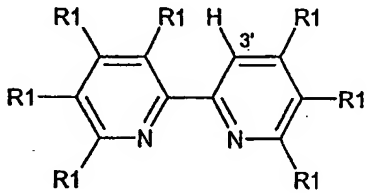
The following biheterocyclyl derivatives may be substituted with one or more R^1 moieties. Thus, a library of the present invention may contain mono-, di-, tri-, tetra-, pentasubstituted biheterocyclyl derivatives. The biheterocyclyl system may be symmetric or asymmetric and they may be symmetricly or asymmetricly substituted with one or more R1 groups.



The 5-membered ring may also be annelated with e.g. a benzene ring:

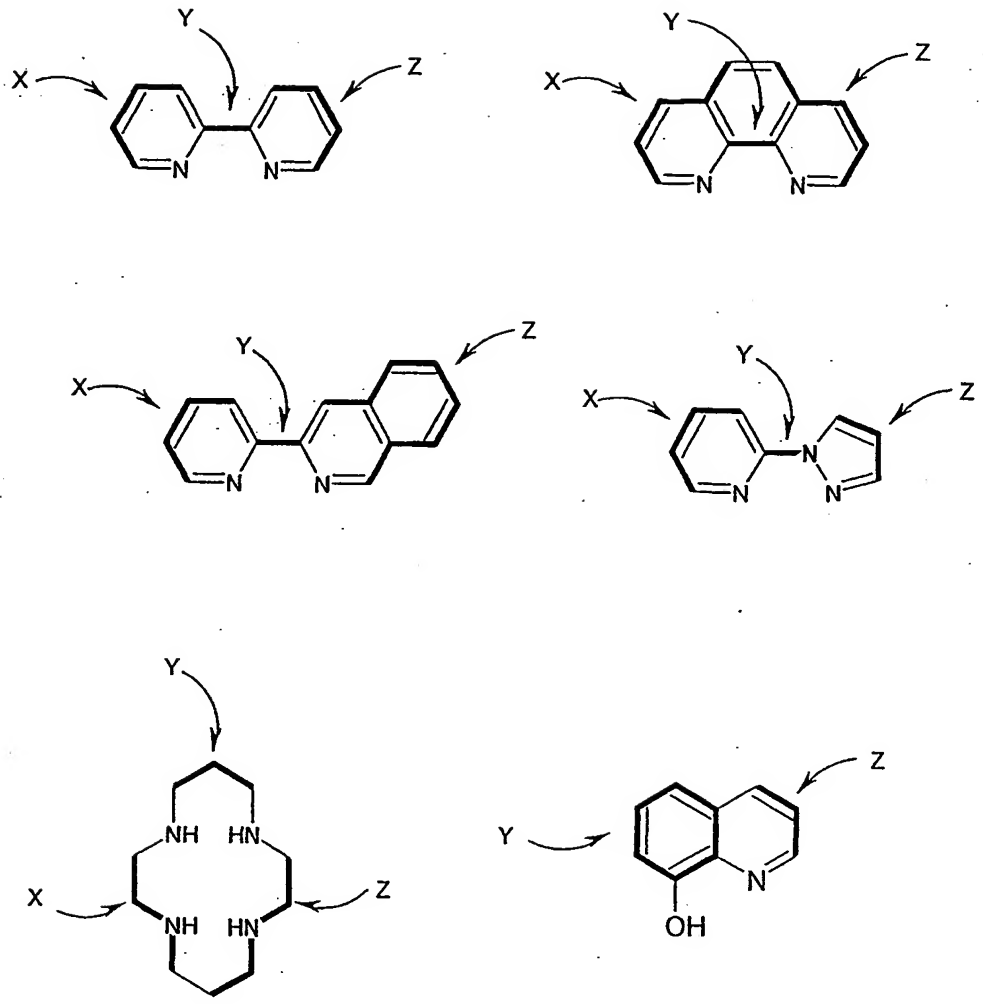


In the figure below, 2,2'-bipyridine is given as an example on a common basic structural element for chemical compounds in a library of the invention, i.e. the 2,2'-bipyridine here functions as the chelator skeleton.

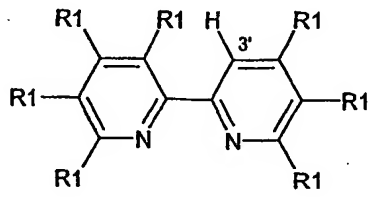


The chemical exemplifications and functionalisation principles given on this skeleton can be applied in analogous manner for other scaffolds with proper adjustments for adoption of suitable chemical routes for the different chelator systems, i.e. Che-R^1 or more specifically Che-A-B-C, wherein Che constitutes the different chelating scaffolds derived from Formula I and described above optionally substituted further with one or more, the same or different, R^1 or more specifically A-B-C groups.

In the following are given some specific structures in which the various elements X, Y and Z are marked with bold.



Thus, a suitable library according to the present invention is a library, that has 2,2'-bipyridine as the same basic structural element. In one embodiment this basic structural element is



Formula II

The construction of libraries according to this invention will be exemplified by the use of 2,2'-bipyridines with no intention to exclude other chelating scaffolds including the general Che-R^1 / Che-A-B-C, wherein Che constitutes the different chelating scaffolds optionally substituted further with one or more, the same or different, R^1 or more specifically A-B-C groups.

Accordingly, the 2,2'-bipyridines of a library according to the invention is normally substituted with one or more functional groups. Thus, a library of the present invention may contain mono-, di-, tri-, tetra-, penta-, hexa- or heptasubstituted bipyridines. The di-, tetra- and/or hexasubstituted bipyridines may be symmetric or asymmetric substituted bipyridines. Normally, up to 4 or at the most 5 substituents are present on the 2,2'-bipyridine skeleton. As seen from the formula II above, the position 3' is preferably substituted with a hydrogen atom.

Libraries containing chemical compounds of the following general formulas are of specific interest in the present context. The libraries are denoted

i) "A-libraries" in those cases where the compounds have a common connecting element, -A-, and

ii) "C-libraries" in those cases where the compounds have a common functional group, –C.

In those cases where the 2,2'-bipyridines are disubstituted, the libraries may be an AA-, AA'-, AC-, CC- or CC'-library (A' is different from A but selected from the same group as A mentioned above; the same applies to C and C').

For trisubstituted 2,2'-bipyridine libraries, the libraries may be an AAA-, AAA'-, AA'A"-, AAC-, AA'C-, ACC-, ACC'-, AC'C"-, CCC-, CCC'- or CC'C"- library (or other possible permutations; the same notation is used as above, i.e. A is different from A', and A and A' are different from A"). The same notation applies for tetra-, penta-, hexa- or heptasubstitued 2,2-bipyridine libraries.

A library according to the invention may be a collection of compounds having e.g. the same A group; it may, however, also be a combination of two individual libraries.

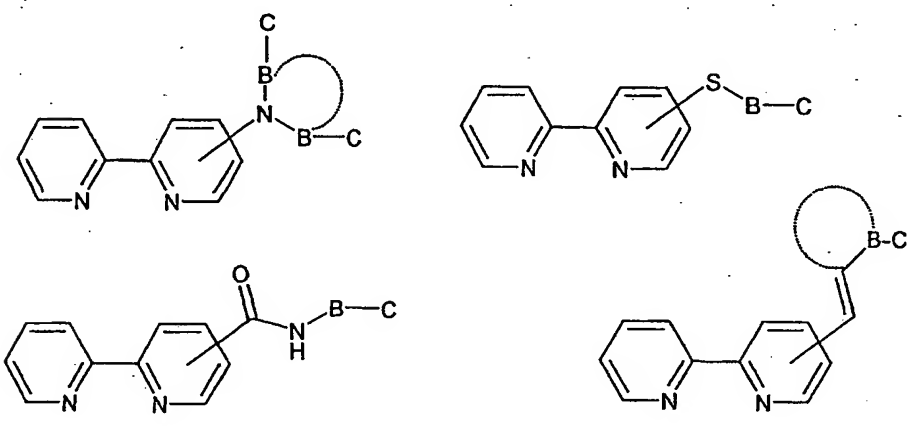
A-libraries

The 2,2'-bipyridines of an A-library have a common connecting group attached directly on the ring system and a variable B-C moiety. Examples on A-libraries are libraries of

compounds according to formula III above in which A is e.g. –O-, –NH-, -S-, -N=, -N<, -CONH-, -CON<, -COO-, -CH=CH- . The functionalisations are made according to well-known chemical reactions with proper considerations of chemical compatibility of the functional groups with respect to the synthetic steps. Some exemplifications will be shown in the following.

Representative examples on A-libraries are

Che-N(B-C)$_2$; Che-S-B-C ; Che-CO-NH-B-C ; Che-CH=CH-B-C ; Che-O-B-C ; Che-NH-CO-B-C ; Che-SO$_2$-NH-B-C as exemplified with the Che being 2,2'-bipyridine :



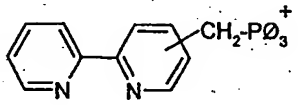
Examples of compounds of an amide library -CONH-B-C, an alkene library -CH=CH-B-C and a retroamide library –NHCO-B-C are detailed in the Experimental part. Note, the B moiety may optionally be part of a ring appended to an exo-cyclic double bond.

The amide library –CONH-B-C can be obtained by reacting a suitably activated carboxylate derivative with appropriate amines as detailed in the Experimental part. The amine library can be obtained by reacting the bipyridyl amines with suitable B-C reagents or sequentially by reaction with a B reagent followed by a C reagent. The alkene library can be obtained by forming the double bond in either direction, i.e. either having the carbonyl moiety on the bipyridyl scaffold or preferably having the carbonyl moiety located on the B moiety as indicated in the example. The thiol library may be obtained by alkylation of the thiol with a B-C reagent or by nucleophilic addition/elimination with a suitable sulphur-containing derivative.

In the formula above, the substituent (e.g. –CONH-B-C) may be positioned anywhere in the 3, 4 or 5 position on the 2,2'-bipyridine skeleton.

An A-library is typically an example of a library that is suitable for use in the randomised approach for optimization.

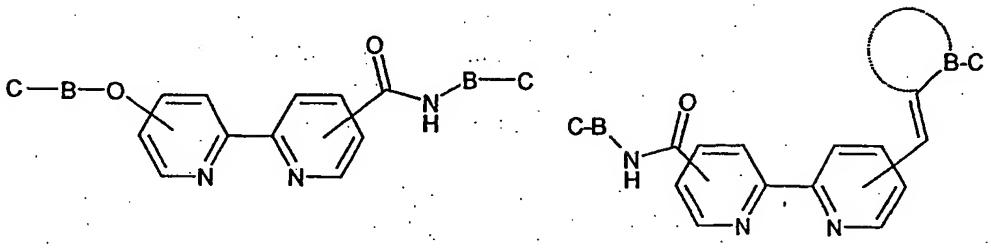
Alkene libraries with different B and C moieties can be obtained by reacting ylides of phosphonium salts or phosphonates such as:



wherein Ø means a phenyl group, with appropriate ketone or aldehyde derivatives as detailed in the Experimental part.

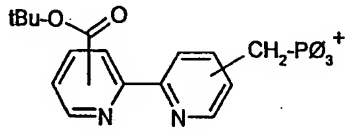
AA'-libraries

Suitable examples on AA'-libraries are
C-B-O-Che-CO-NH-B-C ;
C-B-NH-CO-Che-CH=CH-B-C ;
C-B-NH-CO-Che-NH-CO-B-C ;
C-B-NH-CO-Che-CO-NH-B-C
as exemplified with Che being 2,2'-bipyridine:



i.e. a disubstituted 2,2'-bipyridines (one ring substituted with a functionalised amide and the other ring substituted with a functionalised ether, –O-B-C or in the other example a functionalised alkene -CH=CH-B-C). Note in the latter case, the B moiety may optionally be part of a ring appended to an exo-cyclic double bond.

Alkene/amide (AA') libraries with different B and C moieties can be obtained by reacting ylides of phosphonium salts or phosphonates containing suitably protected carboxylic functions such as:

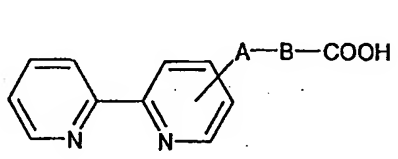


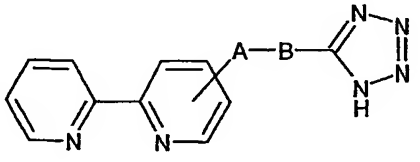
wherein Ø means a phenyl group, with appropriate ketone or aldehyde derivatives, followed by deprotection, activation and coupling with suitable amines as shown for the amide A-library (see Experimental section).

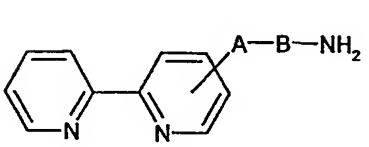
C-libraries

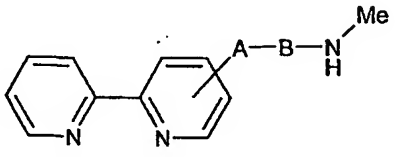
The 2,2'-bipyridines of a C-library have a common functional group either directly attached on the 2,2'-biyridine skeleton or at a position at a distance from the skeleton. Irrespective its position, a characteristic feature of a C-library is that the common functional group is not further derivatized or substituted. Examples on C-libraries are libraries containing 2,2'-bipyridines of formula II wherein R^1 is –A-B-C, -A-C, -B-C or –C (and, if present, B may be further substituted with one or more C groups). Examples on such functional end groups are e.g. –CHO, -NH_2, -$NHCH_3$, -guanidin, -tetrazol, -COOH, -COONa, -$CONH_2$, -NO_2, -CN, i.e. Che-A-B–CHO, Che-A-B-NH_2, Che-A-B-$NHCH_3$, Che-A-B-guanidin, Che-A-B-tetrazol, Che-A-B-COOH, Che-A-B-COONa, Che-A-B-$CONH_2$, Che-A-B-NO_2, Che-A-B-CN.

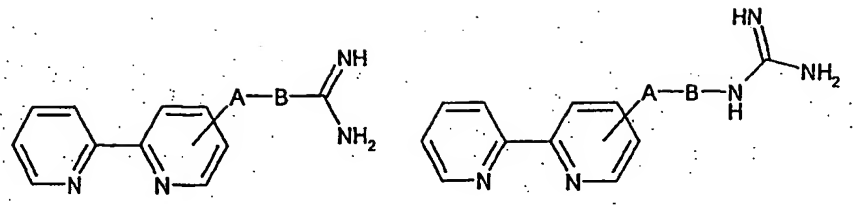
Accordingly, the following formulas represent compounds of C libraries exemplified with Che being 2,2'-bipyridine:







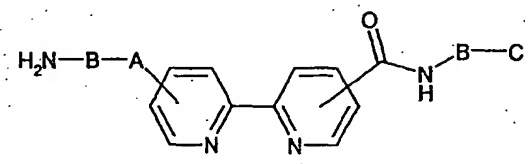




C-libraries are suitable for use in the targeted optimisation approach.

AC-libraries

Examples on an AC-library are
Me_2N-B-A-Che-NH-CO-B-C;
HOOC-B-A-Che-CO-NH-B-C
as exemplified by Che being 2,2'-bipyridine:



Some of the chemical compounds of the above-mentioned formulas are already known and are commercially available or may be prepared according to methods known by a person skilled in the art. However, those chemical compounds that are novel are subject to specific embodiments of the present invention and they may be prepared by the following method:

The present invention also relates to symmetric disustituted bipyridines, i.e. the bipyridine skeleton has been substituted in the same position in the two pyridine rings. The substituent may be the same or different and it may represent the same or different functional group.

Metal ions forming the complex with the heteroalkyl or heterocyclyl moiety in the chemical compounds may advantageously be selected from metal ions, which have been tested for or are used for pharmaceutical purposes.

Such metal ions belong to the groups denoted light metals, transition metals, post-transition metals or semi-metals (according to the periodic system).

The metal ion is typically selected from the group consisting of aluminium, antimony, arsenic, astatine, barium, beryllium, bismuth, boron, cadmium, calcium, cerium, caesium, chromium, cobalt, copper, dysprosium, erbium, europium, gadolinium, gallium, germanium, gold, hafnium, holmium, indium, iridium, iron, lanthanum, lead, lutetium, magnesium, manganese, mercury, molybdenum, neodymium, nickel, niobium, osmium, palladium, platinum, polonium, praseodymium, promethium, rhenium, rhodium, rubidium, ruthenium, samarium, scandium, selenium, silicon, silver, strontium, tantalum, technetium, tellurium, terbium, thallium, thorium, thulium, tin, titanium, tungsten, vanadium, ytterbium, yttrium, zinc, zirconium, and oxidation states and isotopes thereof; in particular aluminium, antimony, barium, bismuth, calcium, chromium, cobalt, copper, europium, gadolinium, gallium, germanium, gold, indium, iron, lutetium, manganese, magnesium, nickel, osmium, palladium, platinum, rhenium, rhodium, rubidium, ruthenium, samarium, silver, strontium, technetium, terbium, thallium, thorium, tin, yttrium, zinc, and oxidation states or isotopes thereof; in particular calcium, cobalt, copper, europium, gadolinium, gallium, iron, magnesium, manganese, nickel, palladium, platinum, ruthenium, samarium, thallium, terbium and zinc (and oxidation states or isotopes thereof, preferably cobalt (II, III), copper (I, II), nickel (II, III), zinc (II) and platinum (0, II, V), palladium (0, II, IV), ruthenium (0, II, III, IV, VI, VIII) or isotopes thereof.

As appears from the examples herein, chemical diverse libraries consisting of various chelating moieties (Che) containing different spacers (A and B moieties) and functionalities (C moities) can be produced and be tested with different metal ions (e.g. Zn, Cu, Ni, Co, Gd, Mn) and bind to different endogenous or engineered binding sites while exhibiting structure-related effects on receptor ligand-induced responses or receptor ligand binding.

Applications in drug discovery processes

As mentioned above, a library according to the present invention is suitable for use in a drug discovery process such as the processes disclosed in PCT/EP00/13389. Such a drug discovery process is described in the following items:

1. A drug discovery process for identification of a small organic compound that is able to

bind to a biological target molecule, the process comprising mutating a biological target molecule in such a way that at least one amino acid residue capable of binding a metal ion is introduced into the biological target molecule so as to obtain a metal ion binding site as an anchor point in the mutated biological target molecule.

2. A drug discovery process according to item 1 further comprising

(a) contacting the mutated biological target molecule with a test compound which comprises a moiety including at least two heteroatoms for chelating a metal ion, under conditions permitting non-covalent binding of the test compound to the introduced metal ion binding site of the mutated biological target molecule, and

(b) detecting any change in the activity of the mutated biological target molecule or determining the binding affinity of the test compound to the mutated biological target molecule.

3. A drug discovery process according to item1 further comprising

(a) contacting the mutated biological target molecule with one or more members of a library of test compounds that comprise a moiety including at least two heteroatoms for chelating a metal ion, under conditions permitting non-covalent binding of at least a member of the library of test compounds to the introduced metal ion binding site of the mutated biological target molecule, and

(b) detecting any change in the activity of the mutated biological target molecule or determining the binding affinity of the test compound to the mutated biological target molecule.

4. A drug discovery process for identification of a small organic compound that is able to bind to a biological target molecule which has at least one metal ion binding site, the process comprising

(a) contacting the biological target molecule with a test compound which comprises a moiety including at least two heteroatoms for chelating a metal ion, under conditions permitting non-covalent binding of the test compound to the metal ion binding site of the biological target molecule, and

(b) detecting any change in the activity of the biological target molecule or determining the binding affinity of the test compound to the biological target molecule.

5. A drug discovery process for identification of a small organic compound that is able to bind to a biological target molecule which has at least one metal ion binding site, the process comprising

(a) contacting the biological target molecule with one or more members of a library of test compounds that comprise a moiety including at least two heteroatoms for chelating a metal ion, under conditions permitting non-covalent binding of at least a member of the library of test compounds to the metal ion binding site of the biological target molecule, and

(b) detecting any change in the activity of the biological target molecule or determining the binding affinity of the test compound to the biological target molecule.

6. A drug discovery process according to any of items1-5 further comprising

(c) identifying the test compound that non-covalently binds to the biological target molecule.

7. A drug discovery process according to any of items1-6 further comprising

(d) selecting two or more test compounds capable of forming a non-covalent binding to a biological target molecule, and capable of changing the activity of the biological target molecule or the binding affinity of the test compound to the biological target molecule

to form a library of test compounds.

8. A drug discovery process according to any of items1-3 or 6-7 further comprising

(e) contacting the biological target molecule in wild-type, non-mutated form with at least one test compound determined to non-covalently bind the mutated biological target molecule in step (a), and

(f) detecting any change in the activity of the biological target molecule or determining the binding affinity of the test compound to the biological target molecule.

9. A drug discovery process according to any of items 1-3 or 6-7 further comprising

(e) contacting the biological target molecule in wild-type, non-mutated form with

two or more members of a library of test compounds, wherein the test compounds in chelated form have been determined to non-covalently bind the mutated biological target molecule in step (a), and

(f) detecting any change in the activity of the biological target molecule or determining the binding affinity of the test compound to the biological target molecule.

10. A drug discovery process according to items 8 or 9 further comprising

(g) identifying the test compound that interacts with the wild-type biological target molecule.

11. A drug discovery process according to any of items 1-7 further comprising

(e) contacting the biological target molecule in wild-type, non-mutated form with at least one test compound determined to non-covalently bind the mutated or non-mutated biological target molecule in step (a) but lacking a metal ion chelated thereto, and

(f) detecting any change in the activity of the biological target molecule or determining the binding affinity of the non-chelated test compound to the biological target moelcule.

12. A drug discovery process according to any of items 1-7 further comprising

(e) contacting the biological target molecule in wild-type, non-mutated form with two or more members of a library of non-chelated test compounds, wherein the test compounds in chelated form have been determined to non-covalently bind the mutated or non-mutated biological target molecule in step (a), and

(f) detecting any change in the activity of the biological target molecule or determining the binding affinity of the non-chelated test compound to the biological target moelcule.

13. A drug discovery process according to items 11 or 12 further comprising

(g) identifying the non-chelated test compound that interacts with the wild-type biological target molecule.

14. A drug discovery process according to any of items 8-13 further comprising

(a) identification of any binding or interaction between the non-chelated test compound and the wild-type biological target molecule.

15. A drug discovery process according to any of items 1-14, wherein the biological target molecule is a protein.

16. A drug discovery process according to item 15, wherein the protein comprises an amino acid residue and wherein the metal ion binding site in the protein is introduced by amino acid substitution at or in the vicinity of

1) a site where the binding of the test compound will interfere with the binding to another protein, for example a regulatory protein, or to a domain of the same protein;

2) a site where the binding of the test compound will interfere with the cellular targeting of the protein;

3) a site where the binding of the test compound will directly or indirectly interfere with the binding of substrate or the binding of an allosteric modulatory factor for the protein;

4) a site where the binding of the test compound may interfere with the intra-molecular interaction of domains within the protein, for example the interaction of a regulatory domain with a catalytic domain;

5) a site where binding of the test compound will interfere with the folding of the protein, for example the folding of the protein into its active conformation; or

6) a site where the binding of the test compound will control the activity of the protein, for example by an allosteric mechanism.

17. A drug discovery process according to any of the preceding items, wherein the metal ion binding amino acid residue in the biological target molecule is introduced by site-directed mutagenesis.

18. A drug discovery process according to any of the preceding items, wherein the mutated biological target molecule is obtained as a recombinant expression product in purified or non-purified form.

19. A drug discovery process according to any of the preceding items, wherein the mutated biological target molecule is obtained as a synthetic or semi-synthetic product.

20. A drug discovery process according to item 15, wherein step (a) in any of items 2-5 comprises the further step of determining, based on the three-dimensional structure of the specific protein in question or the primary structure of the specific protein together with a three-dimensional model of the class of proteins to which the specific protein belongs, the location of the metal ion binding amino acid residue in the mutated or non-mutated protein, and determining the location of at least one other amino acid residue in the vicinity of the metal ion binding amino acid residue.

21. A drug discovery process according to item15, wherein the binding of the test compound to the mutated or non-mutated protein in step (a) in any of items 2-5 is determined using detection of any changes in the biological activity of the protein, competition with binding of a labelled ligand of the protein, or using a metal ion chelator which is in itself detectable or labelled with a detectable labelling agent.

22. A drug discovery process according to item 19, wherein the amino acid residue in the vicinity of the metal ion binding amino acid residue is one which is capable of directly or indirectly binding at least one functional group of the test compound other than the metal ion.

23. A drug discovery process according to item 22, wherein the amino acid residue capable of binding at least one functional group of the test compound other than the metal ion is detected using site-directed mutagenesis of at least one amino acid residue of the protein potentially involved in interaction with said functional group of the test compound other than the metal ion, followed by expression of the mutated protein in a suitable cell, contacting said cell or a portion thereof including the mutated protein with the test compound, and detecting any changes in the activity of the protein, determining any effect on binding in a competitive binding assay using a labelled ligand of the protein, or using a chelating agent which is in itself detectable or labelled with a detectable labelling agent.

24. A drug discovery process according to item 22, wherein the amino acid residue capable of binding at least one functional group of the test compound other than the metal ion is detected by structural analysis employing i) a process involving crystallisation followed by X-ray, or ii) a process involving NMR.

25. A drug discovery process according to item 15, wherein step (a) of any items 2-5 comprises the further steps of improving the binding affinity of a metal ion chelate to the mutated or non-mutated protein, the method comprising

(i) selecting a metal ion chelate with an activity to or a binding affinity to the mutated protein of 50 μM or better as identified by the method of item 21,

(ii) mapping the site of the protein to which the chelate binds using the method of item 20, 23 and/or 24,

(iii) optionally locating at least one amino acid residue in the vicinity of the chelate,

(iv) altering one or more functional group of the chelate to optimise for direct or indirect interaction with said amino acid residue to generate a library of chelate derivatives,

(v) screening the derivatives of step (iv) by the method of item 21,

(vi) selecting metal ion chelates having at least a two fold increase in activity or in binding affinity,

(vii) optionally repeating any one or a combination of two or more of steps (i)-(vi) one or more times to generate metal ion chelating compounds with an improved binding affinity for the mutated or non-mutated protein, and

(viii) optionally screening the thus selected metal ion chelates for binding to the wild-type protein by the method of item 21,

(ix) optionally selecting metal ion chelates having at least an activity or a binding affinity to the wild-type protein of 50 μM or better as identified by the method of item 21, and

(x) optionally repeating any one or a combination of two or more of steps (viii)-(ix) one or more times to generate metal ion chelating compounds with an improved binding affinity for the wild-type protein.

26. A drug discovery process according to item 15, wherein step (e) in any of items 8-12 comprises the further steps of generating a library of test compounds which are derivatives of a test compound found to interact with the wild-type protein in step (e), each test compound in the library being provided with at least one functional group for direct or indirect interaction with at least one amino acid of the wild-type protein, which functional group differs from at least one functional group of each of the other test compounds, and screening the test compound library for compounds interacting with the wild-type protein.

27. A drug discovery process according to item 15, wherein step (e) in any of items 8-12 is performed by detecting any changes in the activity of the protein, detecting an effect on binding in a competitive binding assay using a labelled ligand of the protein, or using a chelating agent which is in itself detectable or labelled with a detectable labelling agent.

28. A drug discovery process according to item 15, wherein step (e) in any of items 8-12 comprises the further step of determining - based on the three-dimensional structure of the specific protein in question or the primary structure of the specific protein together with the three-dimensional model of the class of proteins to which the specific protein belongs, and based on the information provided by the method of item 25 of the location of amino acid residues in the vicinity of the metal ion binding residue introduced in the mutated protein - the location of an amino acid residue in the wild-type protein binding at least one functional group of a test compound.

29. A drug discovery process according to item 28, wherein the amino acid residue capable of binding at least one functional group of the test compound is detected using site-directed mutagenesis of at least one amino acid residue of the wild-type protein potentially involved in interaction with said functional group of the test compound, followed by expression of the mutated protein in a suitable cell, contacting said cell or a portion thereof including the mutated protein with the test compound, and determining any effect on binding using detection of any changes in the biological activity of the protein, a competitive binding assay using a labelled ligand of the protein, or using a chelating agent which is in itself detectable or labelled with a detectable labelling agent.

30. A drug discovery process according to item 28, wherein the amino acid residue capable of binding at least one functional group of the test compound other than the metal ion is detected by structural analysis employing i) a process involving crystallisation followed by X-ray, or ii) a process involving NMR.

31. A drug discovery process according to any of items 1-14, wherein the biological target molecule is selected from the group consisting of proteins, polypeptides, oligopeptides, nucleic acids, carbohydrates, nucleoproteins, glycoproteins, glycolipids, lipoproteins and derivatives thereof.

32. A drug discovery process according to item 31, wherein the biological target molecule is a protein selected from the group consisting of membrane receptors, signal transduction proteins, scaffolding proteins, nuclear receptors, steroid receptors, intracellular receptors, transcription factors, enzymes, allosteric enzyme regulator proteins, growth factors, hormones, neuropeptides or immunoglobulins.

33. A drug discovery process according to item 32, wherein the protein is a membrane protein.

34. A drug discovery process according to item 33, wherein the biological target molecule is a membrane protein and the metal ion binding site in the biological target molecule is introduced in a ligand binding crevice of the membrane protein.

35. A drug discovery process according to item 33, wherein the membrane protein is an integral membrane protein.

Furthermore:

36. A method of identifying a metal ion binding site in a biological target molecule, the method comprising

(a) contacting the biological target molecule with a test compound which comprises a moiety including at least two heteroatoms for chelating a metal ion, under conditions permitting non-covalent binding of the test compound to the biological target molecule, and

(b) detecting any change in the activity of the biological target molecule or determining the binding affinity of the test compound to the biological target molecule.

37. A method of identifying a metal ion binding site in a protein, the method comprising

(a) analysing the nucleotide sequence of the gene coding for the protein in order to deduce the amio acid sequence,

(b) building a molecular model of the protein or a part of the protein based on the deduced amino acid sequence and the generic three-dimensional model of the class of proteins to which the specific protein belongs,

(c) identifying the presence of amino acid residues such as, e.g., histidine, cysteine and/or aspartate residues, capable of binding a metal ion and located in suitable relative positions.

38. A method according to item 36 or 37, wherein the test compound is contacted with two or more biological target molecules for identification of possible metal ion binding sites thereof.

39. A method of identifying a metal ion binding site in a protein, the method comprising

(a) selecting a nucleotide sequence suspected of coding for a protein and deducing the amino acid sequence thereof,

(b) expressing said nucleotide sequence in a suitable host cell,

(c) contacting said cell or a portion thereof including the expressed protein with a

test compound which comprises a moiety including at least two heteroatoms for chelating a metal ion, under conditions permitting non-covalent binding of the test compound to the protein, and detecting any change in the activity of the protein or determining the binding affinity of the test compound to the protein, and

(d) determining, based on the generic three-dimensional model of the class of proteins to which the protein or suspected protein belongs, at least one metal ion binding amino acid residue located in said protein to locate the metal ion binding site of said protein.

40. A method of mapping a metal ion binding site of a protein, the method comprising

contacting the protein with a test compound which comprises a moiety including at least two heteroatoms for chelating a metal ion, under conditions permitting non-covalent binding of the test compound to the protein, and detecting any change in the activity of the protein or determining the binding affinity of the test compound to the protein, and

(b) determining, based on the primary structure of the specific protein in question and the generic three-dimensional model of the class of proteins to which the specific protein of step (a) belongs, at least one metal ion binding amino acid residue located in the membrane protein to identify the metal ion binding site of said membrane protein.

Further applications

The chemical compounds identified according to the methods disclosed in PCT/EP00/13389 and optimized according to the present invention will find use as e.g. drug compounds with abortifacient, acromegalic, alcohol deterrent, amebicidic, anabolic, analeptic, analgesic, anesthetic, antiacne, antiallergic, ophthalmic, anti-Alzheimer's disease, antianginal, antiarrhythmic, antiarthritic, antiasthmatic, antibacterial, antibiotic, anticancer, anticholelithogenic, anticoagulant, anticonvulsant, antidepressant, antidiabetic, antidiarrheal, antiemetic, antiepileptic, antiestrogen, antifungal, antiglaucoma, antihistamine, antihypertensive, antiinflammatory, antilipidemic, antimalarial, antimigraine, antinauseant, antineoplastic, antiobesity, antiparasitic, antiparkinsonian, antiperistaltic, antiprogestogen, antiprolactin, antiprostatic hypertrophy, antipsoriatic, antipsychotic, antirheumatic, antisecretory, antiseptic, antispasmodic, antithrombotic, antitussive, antiulcer, antiviral, anxiolytic, bronchodilator, calcium regulator, cardioprotective, cardiostimulant, cardiotonic, cephalosporin, cerebral vasodilator, chelator, choleretic chrysotherapeutic, cognition enhancer, congestive heart failure, coronary vasodilator,

cystic fibrosis, cytoprotective, dependence treatment, diuretic, dyslipidemia, enzyme, expectorant, fertility enhancer, fibrinolytic, gastroprokinetic, Gaucher's disease, growth hormone, growth hormone insensitivity, haemophilia, heart failure, hematologic, hematopoetic, hemostatic, hepatroprotective, hormone, hyperphenylalaninemia, hyperprolactinemia, hypertensive, hypnotic, hypoammonnuric, hypocalciuric, hypocholesterolemic, hypoglycemia, hypolipaemic, hypolipidemic, idiopathic hypersomnia, immunomodulator, immunostimulant, immunosuppressant, beta-lactamase inhibitor, leukopenia, lung surfactant, mucolytic, muscle relaxant, multiple sclerosis, muscle relaxant, narcotic antagonist, nasal decongestant, neuroleptic, neuromuscular blocker, neuroprotective blocker, neuroprotective, nootropic, non-steroid antiinflammatory disease disease (NSAID), osteoporosis, Paget's disease, platelet aggregation inhibitor, platelet antiaggregant, pneumonia, precocious puberty progestogen, protease inhibitor, psychostimulant, 5-alpha-reductase inhibitor, respiratory surfactant, subarachnoid hemorrhage, thrombolytic, ulcerative colitis, urolithiasis, urologic, vasoprotective, vulnerary and wound healing properties. Important proteins for the present purpose are proteins, which may be stabilised in an active or inactive conformation by a biologically active substance. In this way, it may be possible to obtain an effect of a test compound of the type described herein irrespective of whether the active site of the protein is known, or whether the structure of the active site has been resolved (e.g. by X-ray crystallisation). Examples of such proteins are enzymes, receptors, hormones and other signalling molecules, transcriptional factors and regulators, intra- or extracellular structural proteins, in particular actins; adaptins; antibodies; ATPases; cyclins; dehydrogenases; GTP-binding proteins; GTP/GDP-exchange factors; GTPase activating proteins; GTP/GDP dissociation inhibitors; chaperones; histones; histone acetyltransferases & deacetyltransferases; hormones and other signalling proteins and peptides; kinases; lipases; major facilitator superfamily proteins; motorproteins; nucleases; polymerases; isomerases; proteases; protease inhibitors; phosphatases; ubiquitin-system proteins; membrane proteins including receptors, transporters and channels; transcription factors and tubulins; preferably membrane receptors; nuclear receptors, zinc finger proteins; proteases, tyrosine kinases and matrix proteins. Other important proteins for the present purpose are proteins whose biological activity is regulated by their cellular targeting and whose biological activity therefore can be modulated by drugs, which alter their cellular targeting with or without altering their actual intrinsic activity.

In the following is given methods for the preparation of suitable chemical compounds for use in the present invention. The invention is further illustrated in the following non-limiting examples.

LEGEND TO FIGURES

Figure 1 shows a schematic depiction of the structure of rhodopsin-like 7TMs with one or two conserved key residues highlighted in each TM: AsnI:18; AspII:10; CysIII:01 and ArgIII:26; TrpIV:10; ProV:16; ProVI:15; ProVII:17.

Figure 2 shows the syn, anti and direct coordination of the metal-ion in carboxylate groups. The percentage of each is shown in brackets, based on the analyses of 67 compounds from Cambridge Structural Database (Carrell et al, 1988).

Figure 3 shows the two tautomeric forms of the neutral imidazole side chain of histidine. Without metal, the NH-epsilon-2 form is predominant (80%), whereas the NH-delta-1 form predominates upon metal-binding (75%).

Figure 4 shows the most common geometric shapes and coordination numbers of metal complexes. The most common motifs are italicised.

Figure 5 relates to the Identification of naturally occuring metal-ion binding site in the 7TM leukotriene LTB4 receptor in Example I.1. The figure shows a whole cell competition binding experiment with COS-7 cells expressing the wild type and mutant variants of the leukotriene LTB4 receptor using [^{3}H]-LTB4 as the radioligand.
Panel A. Affinity of Cu(II), 2,2'-bipyridine and the complex therof in the wild type LTB4 receptor.
Panel B. Affinity of Cu(biprydine) in mutant forms of the LTB4 receptor in which the metal-ion binding is severely imparired.
Panel C.Helical wheel diagram illustrating the transmembrane segments of the LTB4 receptor. The two cysteine residues within the transmembrane segment III which have been identified as critical for metal-ion chelator complex binding, Cys93 and Cys97 are indicated in dark gray.

Figure 6 relates to identification of a naturally occurring metal-ion binding site in the 7TM Galanin receptor-1 in Example I.2.
Panels A-F. Mutational analysis and identification of putative Zn(II) chelating residues in the Gal-R1.
Panels G. Model of the Gal-R1 receptor with putative Zn(II) chelating residues shown in the transmembrane segments.
Panel H and I. Model for chelation of Zn(II) by GalR1 from the mutational analysis.

Figure 7 relates to identification of naturally occurring metal-ion chelator binding site in the 12TM dopamine transporter in Example I.3. The figure shows a competition analysis of uptake of [^{3}H]-dopamine in whole COS-7 cells expressing the dopamine transporter.
Panel A. Uptake of [^{3}H]-dopamine by the wild-type dopamine transporter in the presence of free metal zinc-ion and zinc in complex with the chelator 2,2'-bipyridine.
Panel B. Dopamine uptake analysis in a mutant form of the dopamine transporter, [H193K], in which binding of the metal-ion complex has been eliminated (Noregaard et al. EMBO J. (1998) 17: 4266-4273).
Panel C. Effect of metal-ion complex formation on the ability to inhibit [^{3}H]-dopamine uptake in the wild-type and [H193K] mutant dopamine transporter.

Figure 8 shows the binding of various metal-ion complexes to a library of inter-helical metal-ion sites engineered into the tachykinin NK1 receptor as described in Example II.1. COS-7 cells expressing various engineered forms of the NK1 receptor were analyzed by competition binding using [^{125}I]-Substance P as radioligand.
Panel A. IC_{50} values for the zinc and copper metal-ions and complexes thereof with the chelators, 2,2'-bipyridine and phenanthroline are presented in the table. N indicated the number of experiments performed.
Panel B. Data obtained using the chelator cyclam are presented for the NK1 mutant in which an inter-helical metal-ion site has been generated through the introduction of the HisV:05;HisVI:24 exchanges.
Panel C. A helical diagram representing the four sets of inter-helical metal-ion sites which appear in Panel A are indicated.

Figure 9 relates to re-engineering of a metal-ion chelator binding site in the 12TM dopamine transporter as described in Example II.2. Dopamine uptake was analysed in COS-7 cells expressing the wild type and mutant forms of the dopamine transporter in competition with the metal-ion chelator complex, zinc(II)-2,2'-bipyridine. The two panels show two forms of re-engineered dopamine transporters in which the ability to bind the metal-ion chelator complexes have been reconstituted following the elimination of the His193 interaction point.

Figure 10 relates to fluorescence measurement of the relative strength of a selection of chelators to chelate Zn(II) in competition with FluoZin-3 as described in Example II.3.

Figure 11shows the structure-activity relationship of metal-ion complexes in the leukotriene LTB4 receptor as described in Example III.1.Competition binding analysis in

COS-7 cells expressing the LTB4 receptor. Binding of [^{3}H]-LTB4 was analysed in the presence of various copper-ion chelator complexes.

Figure 12 shows the structure-activity relationship of antagonist metal-ion complexes in the metal-ion site engineered tachykinin NK1 receptor as described in Example III.2 Binding of [^{125}I]-Substance P was analysed in COS-7 cells expressing NK1 receptor which have been engineered to bind the zinc metal-ion. Ligand binding is presented in competiton with the zinc metal-ion, the zinc-1,10-phenanthroline complex and with other zinc-chelator xomplexes as indicated.

Figure 13 relates to engineering of *agonistic* metal-ion bindng sites in the Beta-2 adrenergic receptor, demonstrating the importance of the specific amino acids defining the site as described in Example III.3.1.
Panel A. Agonistic metal-ion binding sites probed with either Cu(II)-(2,2'-bipyridine)3 or Cu(II)-(1,10-phenanthroline)3 demonstrating the importance of the specific amino acids composing the site in defining the potency of the sites.
Panel B. Histogram showing the observed efficacy using copper-complexes of 2,2'-bipyridine or 1,10-phenanthroline on selected engineered agonistic metal-ion sites in the Beta-2 adrenergic receptor demonstrating the importance of the observed efficacy on the specific amino acids composing the site. See also figure 20.

Figure 14 shows the structure-activity relation ship of agonistic metal-ion complexes in the metal-ion site engineered beta-2-adrenergic receptor as described in Example III.3.2
The effect of Cu(II) and copper-chelator complexes on stimulation of accumulation of intracellular cAMP was analyzed in COS-7 cells expressing the beta2-adrenoceptor.
Panel A. Washing experiment demonstrating the reversibility of the stimulatory action of the metal-ion complexes.
Panel B. Dose-response analysis of selected copper-chelator complexes on the [F289C;N312C] beta2-AR.

Figure 15 (cont. of figure 14) shows the structure-activity relation ship of agonistic metal-ion complexes in the metal-ion site engineered Beta-2-adrenergic receptor.
The effect of Cu(II)-chelator complexes on stimulation of accumulation of intracellular cAMP was analyzed in COS-7 cells expressing the beta2-adrenoceptor.
Panel C. Testing a library of Cu(II)-2,2'-Bipyridine complexes at 10 micromolar on the [[F289C]-Beta-2 Adrenergic receptor for their efficacy in stimulating cAMP. For a list of compounds see the list below.

Figure 16 shows the structure-activity relationship of antagonistic metal-ion complexes in a soluble protein, the enzyme factor VIIa as described in Example III.4. The figure shows a comparison of selected metal-ion complexes on the binding of [3H]-LTB4 and the inhibition of the enzymatic activity of the active form of Factor VII (FVIIa) in COS-7 cells expressing respectively the LTB4 receptor (Panel B) and the FVIIa (Panles A and C). For stucture of the chelators see the list below.

Figure 17 shows a structure-based optimization of metal-ion chelators for secondary interactions in the CXCR4 receptor and other biological targets as described in Example III.5 Helical wheel diagram for the CXCR4 receptor. The Asp171 residue present in the transmembrane segment IV, and which is considered a major attachment site for the binding of the cyclam chelator is shown in white on black. Positions, which in combination are proposed to constitute putative metal-ion binding sites, are high-lighted in pairs and in black on dark gray.

Figure 18 relates to affinity optimization of metal-ion chelators in the LTB-4 leukotriene receptor as described in Example III.6.
A library of approximately 500 substituted 2,2'-bipyridines were tested in competition binding with [^{3}H]-LTB4 in COS-7 cells expressing the LTB4 receptor. From this test, TM-369 was identified as having a 50-fold increased affinity compared to the unsubstituted 2,2'-bipyridine scaffold.

Figure 19 relates to probing different metal-ions in an engineered Bis-His TM-V Kappa opioids.

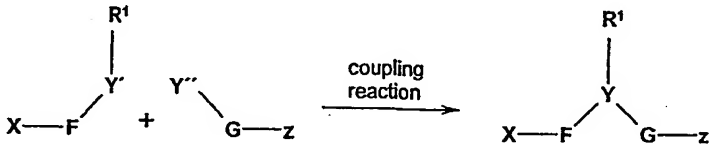
Figure 20 shows the dependency of amino acids defining the metal-ion site, metal-ion or metal-ion chelator on the observed efficacy in agonist metal-ion binding sites in the beta-2 adrenergic receptor.
Shown is the cAMP production in response to 100 micromolar complex, compound, free ion (10 micromolar Cu(II)) or 1 micromolar pindolol.

Figur 21 is related to Exampel III.7. The figure illustrates the establishment of increased affinity in a silent metal-ion site engineered receptor through second-site interaction obtained by side-chain modification of a stable metal-ion chelator complex to be used in a genetically modified animal. Signal transduction is determined as accumulation of [3H] Inositol triphosphat in COS-7 cells expressing either the wild-type RASSL receptor (called RO2 in the figure) or the metal-site engineered RASSL receptor (called CysVII:06 in the

figure). The metal ion site is located between position TM III:08 (a natural Asp residue) and VII:06 (an engineered Cys residue). The receptors are stimulated with a constant dose of the non-peptide agonist ICI 199,441 to a sub-maximal level and inhibitory dose-response experiments are performed with preformed stable Pd(II) complex with either 4,4-dimethyl-bipyridine (chemical structure shown to the upper right and dose-response experiments in wild-type receptor and metal-ion site engineered receptor are shown in the panel to the upper left) or in complex with compound 433 (chemical structure shown to the lower right and dose-response experiments in wild-type receptor and metal-ion site engineered receptor are shown in the panel to the lower left) – in both cases acetate (AcO) was used as leaving group.

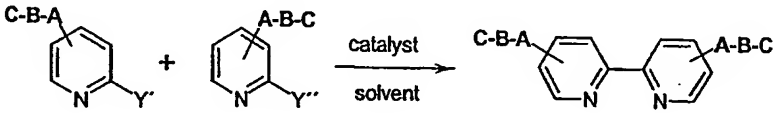
Formula I may be constructed by well-known synthetic steps involving coupling reactions, including Stille-, Suzuki-, Negishi-, Ullmann-couplings (C-C bond formations), condensation reactions, including heterocyclic ring-forming reactions, elimination reactions, cycloaddition reactions, and/or substitution reactions known from the common literature, as illustrated with some typical but non-limiting reaction schemes.
The usual considerations regarding which functional groups that are compatible with the different types of chemistries should always be taken into account when selecting synthetic routes, order of introduction of functional groups and their interconversions, etc, which accordingly will differ on a case by case basis but are evident for the skilled person.

One typical connection of coordinating moieties is depicted in Scheme I, where Y′ and Y″ are defined such that they represent functional groups enabling coupling reactions.



Scheme I

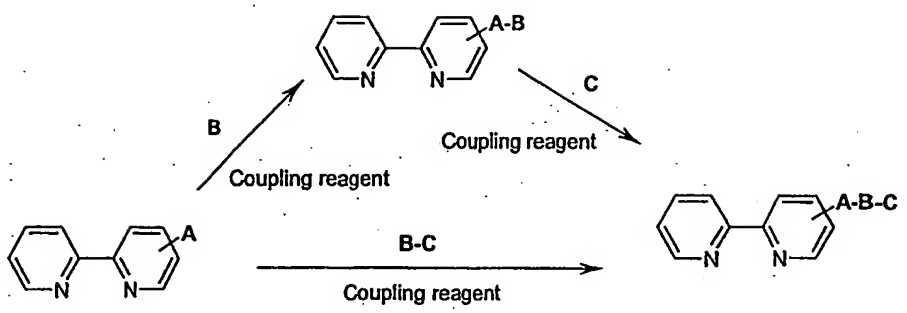
More specific descriptions of the reaction types are exemplified in Schemes II, III and IV respectively. Scheme II illustrates the C-C-bond forming reaction in the 2,2'-bipyridine series.



Reaction type I

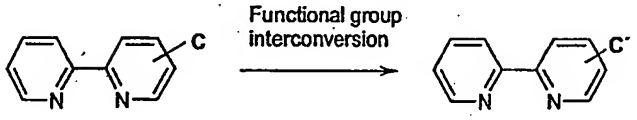
Scheme II

Modification of the chelating scaffolds exemplified by bipyridins can be made in essentially two ways, depicted in Schemes III and IV, either by coupling of an A-moiety with a B-moiety followed by C-moiety, or a B-C-moiety, or as illustrated in Scheme IV by a functional group interconversion.



Reaction type II

Scheme III

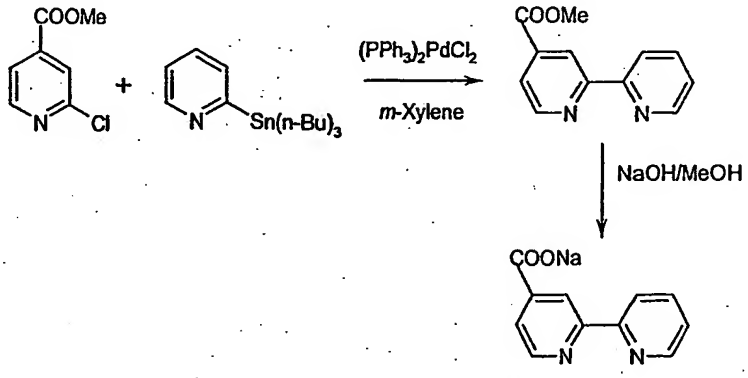


Reaction type III

Where **C** and **C'** represent a change in functionality.

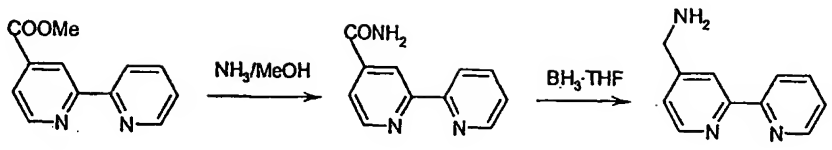
Scheme IV

Coupling of functionalised heterocyclic ring systems such as chloropyridines with trialkyl tin pyridines can be performed by the Stille coupling method, and exemplified in Scheme V.



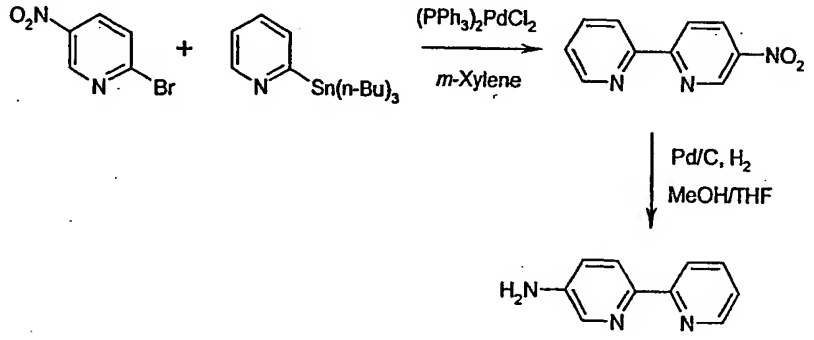
Scheme V

Typical functional group interconversions are exemplified by transforming -$COOCH_3$ into a –CH_2-NH_2 moiety as exemplified with the 2,2'.bipyridine system.



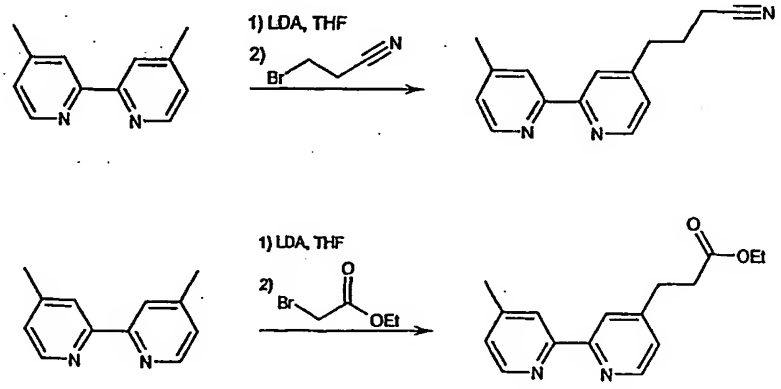
Scheme VI

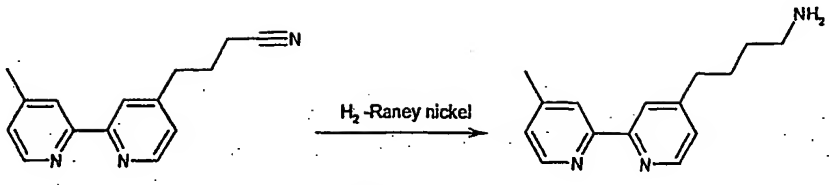
Certain other types of functionalities on the pyridine ring can accepted in the coupling reaction step as illustrated in Scheme VII.



Scheme VII

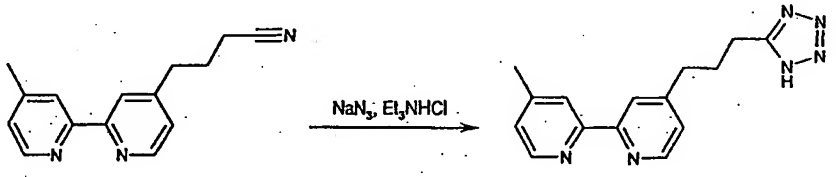
Other types of functionalisations are illustrated by the synthesis of longer chain 2,2´-bipyridyl amines from the symmetric dimethyl-2,2´- bipyridines, by generation of dimethyl-2,2 bipyridine anion with LDA followed by addition of the appropriate electrophile. Standard reduction of the nitrile yielded the desired product as outlined in Scheme VIII.





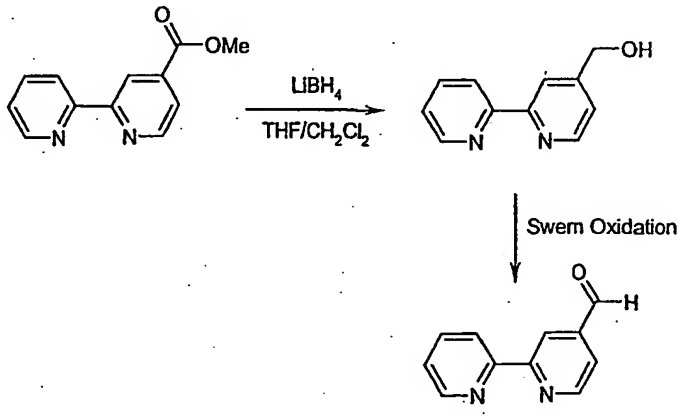
Scheme VIII

Functional group interconversions could utilise common intermediates (cf. Schemes VIII and IX) as illustrated by the bipyridine functionalised chelating scaffold.



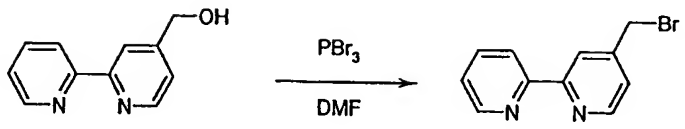
Scheme IX

Reduction of the bipyridine esters were performed by using $LiBH_4$, in DCM/THF as solvent, whereupon the corresponding alcohols were oxidised under Swern conditions to the corresponding aldehydes, as exemplified in Scheme X.



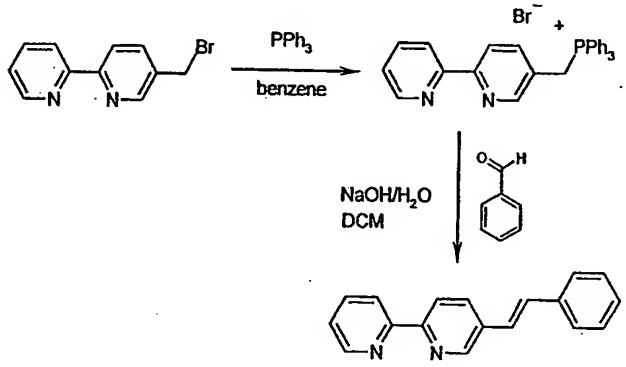
Scheme X

Functional group interconversion of the methylhydroxy functionality to the corresponding bromide can be performed by standard literature procedure as seen in Scheme XI.



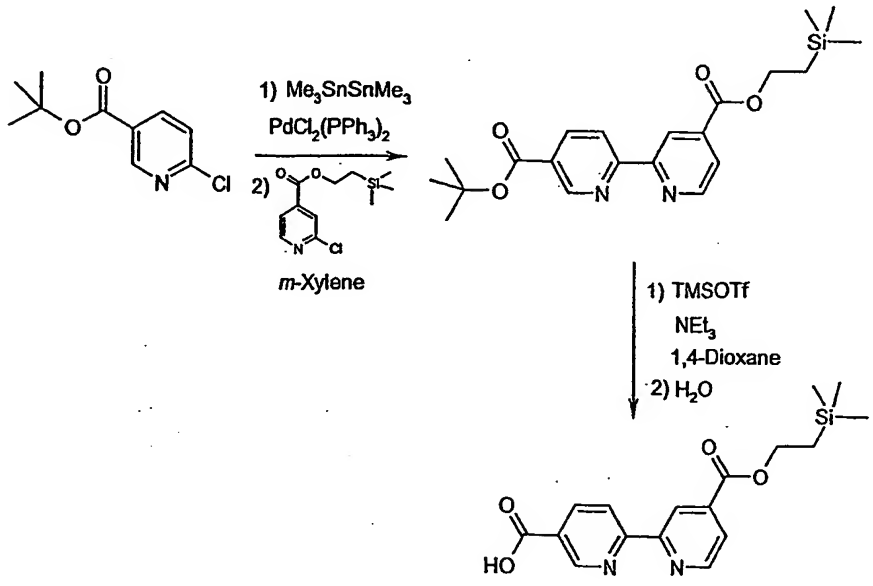
Scheme XI

The synthesis of alkenes the Wittig reaction protocol was utilised as outlined in Scheme XII.

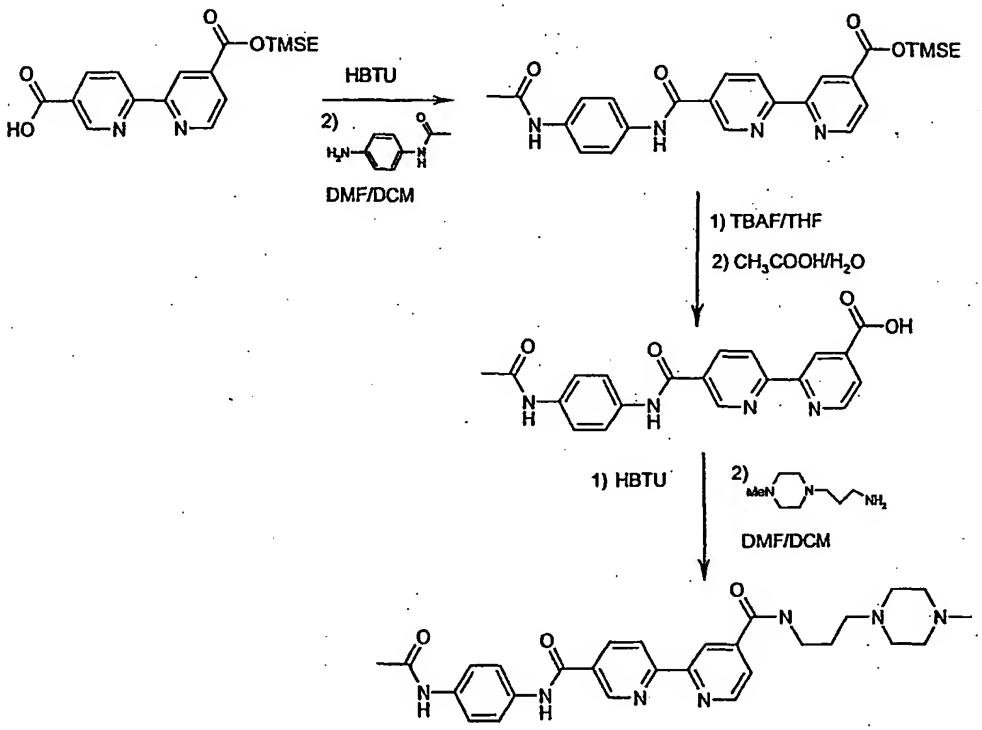


Scheme XII

Coupling of functionalised chloropyridines were performed by using $Me_3SnSnMe_3$, and thereby in situ forming the corresponding trimethyltin pyridine, which was subsequently coupled to the differently substituted chloropyridine as shown in Scheme XIII.

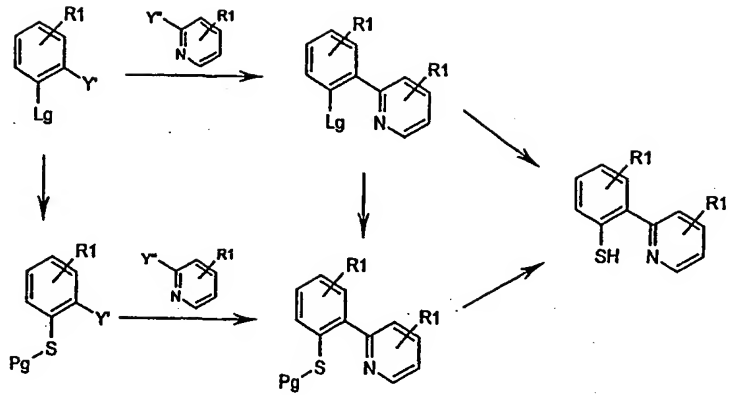


Further functionalisations of the unsymmetrically substituted bipyridines were performed by an orthogonal deprotection procedure as in Scheme XIV using standard literature procedure. Amine coupling of the free carboxyl acids can be performed by using a suitable coupling reagent.



Scheme XIV

Similarly, other chelator systems may be formed and manipulated. As an example on a chelator which have one of the coordinating atom(s) outside the ring system is 2-(2-pyridyl)thiophenol (See Scheme XV). In this case, the construction may follow different routes, i.e. the coordinating atoms may be introduced at various stages, protected or unprotected, schematically illustrated in Scheme XV.



Scheme XV

Further functionalisation of the R1-group can be made analogous to the above-described procedures.

Abbreviations.

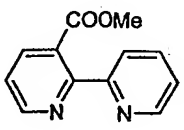
DCM	Dichloromethane
DIBAL	Diisobutylaluminum hydride
DMF	*N,N'*-Dimethylformamide
DMSO	Dimethylsulfoxide
EDC	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
HBTU	*O*-Benzotriazole-1-yl-*N,N,N', N'*-tetramethyluronium hexafluorophosphate
HOBT	1-Hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
LDA	Lithium diisopropylamide
Lg	Leaving group
MS	Mass Spectrometry
NMR	Nuclear magnetic resonance
Pg	Protecting group
R.T.	Room temperature
TBAF	Tetrabutylammonium fluoride
Tf	Triflate
TFFH	Fluoro-*N,N,N'*, -tetramethylformamidinium

THF	Tetrahydrofurane
TLC	Thin Layer Chromatography
TMS	Trimethylsilyl
TMSE	2-(Trimethylsilyl)-ethyl

General chemical procedures

All reagents/chemicals were used as received unless otherwise noted. Methyl esters of 2-chlorocarboxy pyridines were synthesised using carbonyldiimidazole. Coupling of the 2-chloromethyl carboxylates with 2-(tributyl)tin pyridine and hydrolysis of the resulting 2,2´-bipyridinemethyl esters were performed by Stille coupling according to the method described by Panetta *et al.* (*J. Org. Chem*, **1999**, *64*, 1015-1021). Coupling between the 2,2´-bipyridinesodium carboxylate and selected primary amines (example 3) were perfomed according to standard procedure. Reduction of 4-nitro-2,2´-bipyridine to the corresponding 4-amino-2,2´-bipyridine was accomplished by hydrogenation according to the method by Imperali *et al.* (*J. Org. Chem.*, **1996**, *61*, 8940-8948). All terazoles of 2,2´-bipyridnes were synthesised according to the method of Koguru *et al.* (SYNTHESIS, **1998**, 910-914). Guanidines of amino or alkylamino 2,2´-bipyridine were synthesised according to the method of Patek *et al.* (SYNTHESIS, **1994**, 579-582). Aldehydes of 2,2´-bipyridine were synthesised by reduction of 2,2´-bipyridinemethyl esters using lithiumborohydride according to the method of Uenishi *et al.* (*J. Org. Chem.*, **1993**, *58*, 4382-4388). Oxidation of the resulting methylhydroxy 2,2´-bipyridine to the corresponding aldehyde was performed according to the method of Swern *et al.* (*Tetrahedron*, **1978**, *34*, 1651-1660). All other reactions were carried out according to reported procedures.

EXAMPLE 1

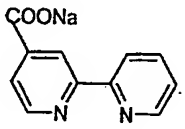


2,2´-Methyl-2,2´-bipyridine-3-carboxylate. 2-Chloronicotinic acid methylester (154.9 mmol, 26.7g) was suspended in 500ml dry *m*-xylene, in an oven dried 1000 ml two-necked round bottomed flask equipped with stirrer magnet. 2-Tributyltin pyridine (176.2 mmol, 80g) was added and thereupon *bis*-triphenylphosphinepalladium chloride (9.6 mmol, 6.4g). The

resulting mixture was heated to 130 C for 6h under N_2-atmosphere. The dark-brown mixture was then allowed to cool to ambient temperature, and the solvent was removed by evaporation *in vacuo*. The residue was mixed with dichloromethane (50 ml), and purified by column chromatography (CH_2Cl_2:EtOH, 95:5). The pure compound was retrieved as white crystals. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.74 (dd, J = 1.68, 5.1 Hz), 8.61-8.59 (m, 1H), 8.15-8.12 (m, 1H), 7.96 (dd, J = 1.71, 7.8 Hz, 1H), 7.87-7.77 (m, 1H), 7.36 (dd, J = 4.71, 9.0 Hz, 1H), 7.32-7.28 (m, 1H), 3.78 (s, 3H).

In the same manner the corresponding ethyl, propyl, isopropyl, isobutyl, *tert*-butyl, phenyl, pentafluormethyl, 9-fluorenylmethyl, 2-trimethylsilylethyl bipyridine esters (in 3, 4 or 5-position), (alkyl)aldehydes or (alkyl)nitriles are synthesised.

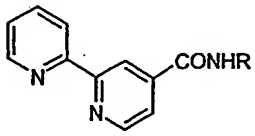
EXAMPLE 2



Sodium-2,2´-Bipyridine-4-carboxylate. Sodiumhydroxide (131.0 mmol, 5.2g) was dissolved in absolute methanol (300 ml). Bipyridine-4-carboxymethyl ester (130.7 mmol, 28g) was added and the resulting mixture was refluxed for 3h. A white precipitate formed. The mixture was allowed to cool to ambient temperature. The white precipitate was collected by filtration, and washed with ether. The mother liquor diluted with ether (150 ml), and the resulting precipitated was collected by filtration and washed with ether. The remaining solid was allowed to dry at room temperature.

Sodium-2,2´-bipyridine-3-carboxylate and sodium-2,2´-bipyridine-5-carboxylate were prepared according to identical procedure.

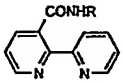
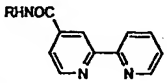
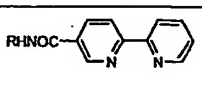
EXAMPLE 3

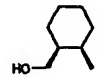
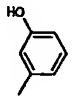
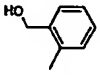
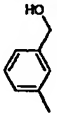
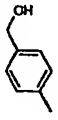
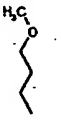
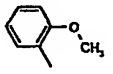
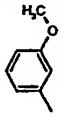
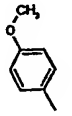


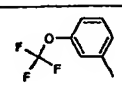
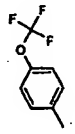
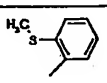
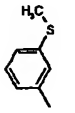
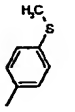
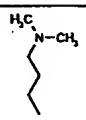
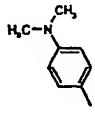
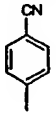
Sodium bipyridinecaboxylate (0.4 mmol, 88.9 mg) was dissolved in 4 ml DMF/CH_2Cl_2 (1:1). Acetic acid (0.4 mmol), coupling reagent (HBTU, TFFH or EDC) (0.4 mmol, 151.7 mg), amine (0.4 mmol) and triethyl amine (0.4 mmol) were added and shaken for 18h at room temperature. The reaction mixture was then quenched with aqueous NaOH (2 ml, 2M), and extracted into 10 ml CH_2Cl_2. The solvent was evaporated at room temperature. Purification was performed either by HPLC (acetonitrile/water in a gradient of acetonitrile in 85 %→ 0 % at a flow rate of 10 ml/min. Column type : YMC-ODS 250x10 mm) or column chromatography on neutral alumina (eluent: acetone/heptane with acetone in a gradient of 20 %→40 %). The desired amides were identified by LC/MS, using 5mM NH_4OAc as the mobile phase, and ES as the ionisation technique.

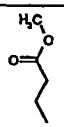
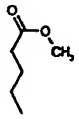
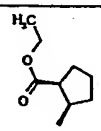
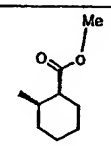
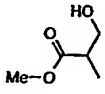
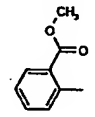
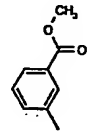
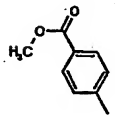
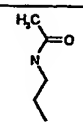
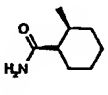
In an analogous manner amides summarised in Table 1 are synthesised.

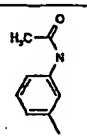
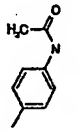
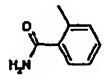
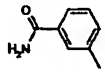
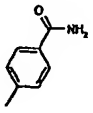
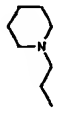
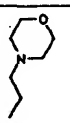
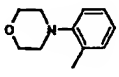
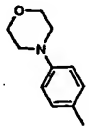
Table 1 2,2'-Bipyridyl amides as a typical "A"-type library.

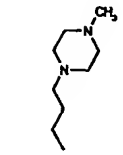
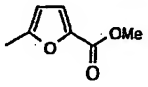
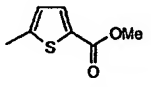
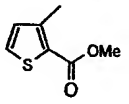
R									
	Coupl. reagent	Analyt. method	Purificat. method	Coupl. reagent	Analyt. method	Purificat. method	Coupl. reagent	Analyt. method	Purificat. method
H	—	NMR	HPLC	HBTU	NMR	HPLC	HBTU	NMR	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC

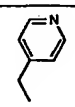
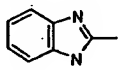
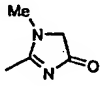
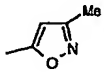
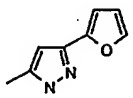
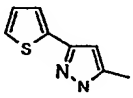
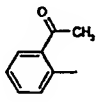
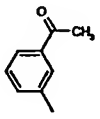
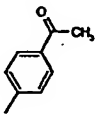
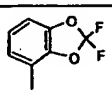
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
				HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
							HBTU	LC-MS	HPLC
				HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
				HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC			
	TFFH	LC-MS	Alumina	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	Alumina	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC

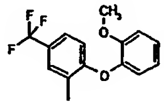
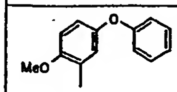
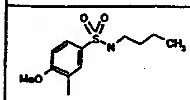
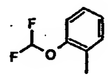
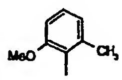
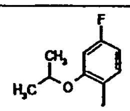
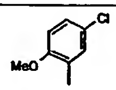
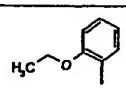
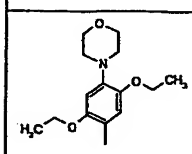
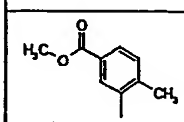
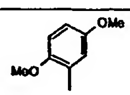
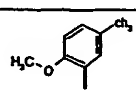
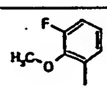
	TFFH	LC-MS	Alumina	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
				HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	Alumina	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	Alumina	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC			
	TFFH	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	Alumina	HBTU	LC-MS	Alumina	HBTU	LC-MS	HPLC
	TFFH	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC

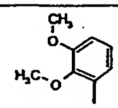
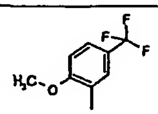
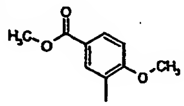
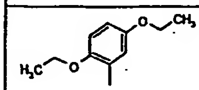
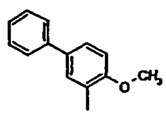
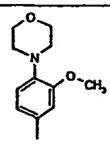
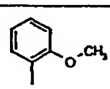
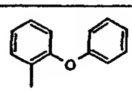
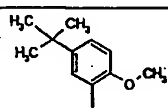
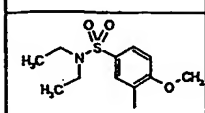
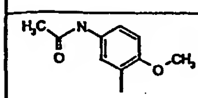
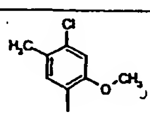
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
				HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC						
	TFFH	LC-MS	Alumina	HBTU	LC-MS	HPLC			
	TFFH	LC-MS	Alumina	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	HPLC	HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
				HBTU	LC-MS	HPLC			

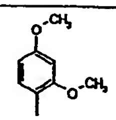
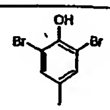
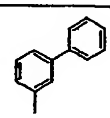
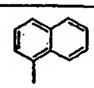
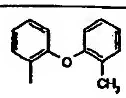
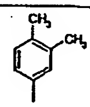
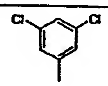
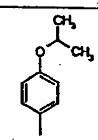
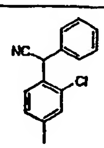
				HBTU	LC-MS	Alumina	HBTU	LC-MS	HPLC
				HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
				HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
				HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	Alumina	HBTU	LC-MS	HPLC	HBTU	LC-MS	Alumina
	TFFH	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC

	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
				HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
				HBTU	LC-MS	HPLC			
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC			
	TFFH	LC-MS	HPLC						
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	HPLC				HBTU	LC-MS	HPLC
				HBTU	LC-MS	Alumina			
							HBTU	LC-MS	HPLC
				HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	Alumina	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
				HBTU	LC-MS	HPLC	HBTU	LC-MS	Alumina
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC			
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC

	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
				HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
							HBTU	LC-MS	HPLC
	TFFH	LC-MS	HPLC						
	TFFH	LC-MS	Alumina						
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	Alumina			
	TFFH	LC-MS	Alumina	HBTU	LC-MS	HPLC			
	TFFH	LC-MS	Alumina	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	TFFH	LC-MS	Alumina	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
	EDC	LC-MS	HPLC	HBTU	LC-MS	HPLC	HBTU	LC-MS	HPLC
				HBTU	LC-MS	HPLC			

				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			

				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			

				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			
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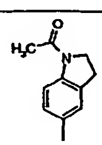
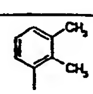
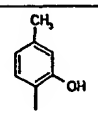
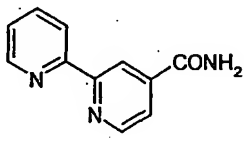
				HBTU	LC-MS	HPLC			
				HBTU	LC-MS	HPLC			

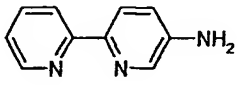
				HBTU	LC-MS	HPLC			



2,2´-Bipyridine-4-carboxamide.

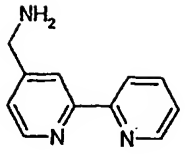
^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.13 (dd, J = 0.75, 2.1 Hz, 1H), 8.72 (ddd, J = 0.96, 5.25, 6.1 Hz, 1H), 8.47-8.42 (m, 2H), 8.36 (dd, J = 3.0, 8.4 Hz, 1H), 8.23 (br. s, 1H), 7.98 (td, J = 1.68, 7.98 Hz, 1H), 7.65 (br. s, 1H), 7.50 (ddd, J = 1.29, 5.1, 7.45 Hz, 1H).

EXAMPLE 4



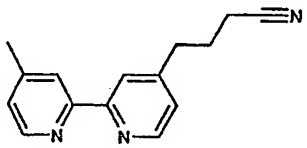
5-Amino-2,2´-bipyridine. 5-Nitro-2,2'-bipyridine (0.641 mol, 129 mg) was dissolved in MeOH/THF (5ml+5ml). To the solution was added Pd/C (5 %, 50 mg) and the reaction mixture was set under an H_2-atmosphere and stirred for 24 h at room temperature. The reaction mixture was filtered through Celite, and the filtrate was evaporated *in vacuo*. The residue was purified by column chromatography (neutral Al_2O_3, 5 % EtOH in DCM), to yield the desired product. Yield: quantitative. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.63-8.61 (m, 1H), 8.27-8.15 (m, 3H), 7.76 (td, J = 1.9, 7.8 Hz, 1H), 7.22 (m, 1H), 7.09 (dd, , J = 2.8, 8.4 Hz, 1H), 3.88 (br. s, 2H).

EXAMPLE 5



4-(Aminomethyl)-2,2´-bipyridine. 2,2'-Bipyridine-4-carboxamide (15.0 mmol, 3.0g) was placed in an oven dried 100 ml round bottomed flask equipped with stirrer magnet. Borane tetrahydrofurane complex (30 mmol, 30 ml, 1M solution in THF) was slowly added, and the content of the flask was stirred for 15 h. The reaction mixture was quenched with saturated NH_4Cl (aq). The mixture was made basic (pH = 9) with 1M NaOH. The resulting mixture was stirred for 1h at room temperature after which the organic phase was separated, and the aqueous phase was extracted twice with EtOAc (2x30 ml). The combined organic phases were dried over $MgSO_4$, and filtered through a sintered glass funnel. The solvent was removed by evaporation *in vacuo.*

EXAMPLE 6



4-(3-Cyanopropyl)-4'-metyl-2,2'-bipyridyl: 4,4'-Dimethyl-2,2'-bipyridyl (5.0 g, 27 mmol) was dissolved in dry THF (50 ml) under a nitrogen atmosphere, in a flame-dried flask, equipped with a stirrer. The solution was cooled to –78 °C, and a solution of LDA (20 ml, 33 mmol) was added. The reaction mixture was allowed to warm to room temperature for 1,5 hours. This solution was cannulated into a solution of 3-bromopropionitrile (3.4 ml, 40 mmol) in dry THF (20 ml) at –78 °C, placed in a flame-dried flask under a nitrogen atmosphere, equipped with a stirrer. The reaction mixture was allowed to reach room temperature slowly over night, and quenched by addition of a saturated aqueous solution of sodium bicarbonate. Extractive work-up using ethyl acetate, drying and evaporation, gave the crude product of major components being starting material and expected product. The crude product was purified by column chromatography (Alumina; EtOAc:Heptane 1:2). Yield: 2.1 g (33 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.02 (m, 2H), 2.32 (t, J = 7.07 Hz, 2H), 2.39 (s, 3H), 2.81 (t, J = 7.63 Hz, 2H), 7.10 (m, 2H), 8.20 (s, 1H), 8.24 (s, 1H), 8.47 (d, J = 5.09 Hz, 1H), 8.54 (d, J = 5.09 Hz, 1H).

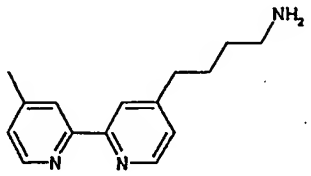
5-(3-Cyanopropyl)-5'-metyl-2,2'-bipyridyl: Same procedure as described above. Yield: 0.67 g (52 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.04 (m, 2H), 2.39 (t, J = 6.97 Hz, 2H), 2.42 (s, 3H), 2.86 (t, J = 7.54 Hz, 2H), 7.68 (d, J = 8.26 Hz, 2H), 8.32 (d, J = 7.91 Hz, 1H), 8.40 (d, J = 8.29 Hz, 1H), 8.53 (s, 2H).

4-(2-Cyanoethyl)-4'-metyl-2,2'-bipyridyl: Same procedure as described above. Yield: 1.17 g (19 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.48 (s, 3H), 2.77 (t, J = 7.35 Hz, 2H), 3.08 (t, J = 7.44 Hz, 2H), 7.22 (m, 2H), 8.30 (s, 1H), 8.37 (s, 1H), 8.56 (d, J = 4.90 Hz, 1H), 8.66 (d, J = 4.98 Hz, 1H).

5-(2-Cyanoethyl)-5'-metyl-2,2'-bipyridyl: Same procedure as described above. Yield: 27 mg (8 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.39 (s, 3H), 2.68 (t, J = 7.25 Hz, 2H), 3.02 (t, J = 7.35 Hz, 2H), 7.59-7.73 (m, 2H), 8.28 (m, 1H), 8.36 (m, 1H), 8.51 (m, 1H), 8.56 (m, 1H).

5-Cyanomethyl-5'-metyl-2,2'-bipyridyl: Same procedure as described above. Yield: 51 mg (15 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.39 (s, 3H), 3.81 (s, 2H), 7.63 (m, 1H), 7.79 (m, 1H), 8.28 (d, J = 8.10 Hz, 1H), 8.40 (d, J = 8.10 Hz, 1H), 8.51 (m, 1H), 8.60 (m, 1H).

EXAMPLE 7



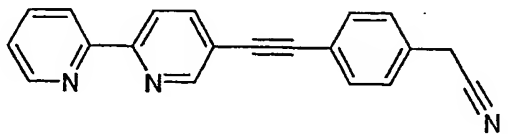
4-(4-Aminobutyl)-4'-methyl-2,2'-bipyridyl: 4-(3-Cyanopropyl)-4'-methyl-2,2'-bipyridyl (125 mg, ca. 0.5 mmol) was dissolved in 96 % ethanol (5 ml) and catalytic amount of Raney nickel was added. The reaction was stirred over night under 1 atmosphere of hydrogen. Evaporated and purified by chromatography (alumina, DCM:MeOH:NH_4OH 95:5:0.5). Yield: 70 mg (58 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.49 (m, 2H), 1.68 (m, 2H), 2.36 (s, 3H), 2.64 (t, J = 7.72 Hz, 2H), 2.68 (s, 2H), 2.70 (t, J = 7.07 Hz, 2H), 7.05 (m, 2H), 8.15 (m, 2H), 8.46 (dd, J = 0.47, 4.99Hz, 1H), 8.48 (dd, J = 0.66, 5.00Hz, 1H).

5-(4-Aminobutyl)-5'-methyl-2,2'-bipyridyl: Same procedure as described above. Yield: 181.4 (44 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.52 (m, 2H), 1.70 (m, 2H), 1.80 (s, 2H), 2.38

(s, 3H), 2.68 (t, J = 7.54 Hz, 2H), 2.74 (t, J = 7.06 Hz, 2H); 7.59 (m, 1H), 7.62 (m, 1H), 8.24 (d, J = 6.03 Hz, 1H), 8.26 (d, J = 8.10 Hz, 1H), 8.48 (s, 1H), 8.49 (s, 1H).

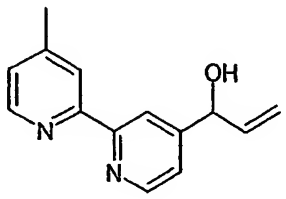
4-(3-Aminopropyl)-4'-methyl-2,2'-bipyridyl: Same procedure as described above. Yield: 190 mg (50 %). ^{1}H NMR (DMSO-d_6; 300 MHz) δ 1.80 (m, 2H), 2.41 (s, 3H), 2.68 (t, J = 7.16 Hz, 2H), 2.74 (t, J = 7.82 Hz, 2H), 4.48 (s, broad, 2H), 7.28 (m, 2H), 8.23 (s, 1H), 8.25 (s, 1H), 8.53 (d, J = 5.08 Hz, 1H), 8.56 (d, J = 5.08 Hz, 1H).

EXAMPLE 8



(4-[2,2']Bipyridinyl-5-ylethynyl-phenyl)-acetonitrile. 4-(2-(2'-Pyridyl)pyridyl)acetylene (0.6 g, 2.0 mmol), iodophenylacetonitril (0.54 g, 2.2 mmol), copperiodide (38 mg, 0.2 mmol), tetrakis(triphenylphosphine)palladium(0) (230 mg, 0.2 mmol) and triethylamine (2.8 ml, 20 mmol) in DMF (10 ml) was stirred at R.T. under nitrogen for 24 hours. The reaction was reduced *in vacuo*, and water and ethylacetate added. The organic layer was dried, reduced *in vacuo* and purified on a silica column, using ethylacetate/ether (1:1) as eluent. Recrystalised in ethylacetate. Yield: 30 mg (5 %). ^{1}H NMR($CDCl_3$, 300 MHz) δ 3.82 (s, 1H), 7.35 (m, 1H), 7.38 (d, J = 8.1 Hz, 2H), 7.61 (d, J = 8.1 Hz, 2H), 7.85 (br t, J = 7.7 Hz, 1 H), 7.97 (dd, J = 8.3 Hz, J = 2.1 Hz, 1H), 8.45 (d, J = 8.1 Hz, 2H), 8.72 (d, J = 4.9 Hz, 1H), 8.85 (m, 1H).

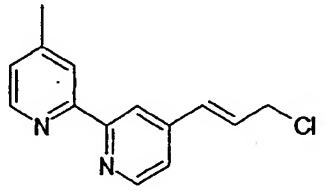
EXAMPLE 9



1-(4'-Methyl-[2,2']bipyridinyl-4-yl)-prop-2-en-1-ol. 4-Formyl-4'-methyl-2,2'-bipyridine (10.1 mmol, 2.0 g) was dissolved in drv tetrahvdrofuran (100 ml) at -20 °C before vinyl

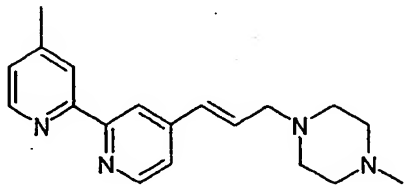
magnesium bromide (12.0 mmol, 1M, 12.0 ml) was added dropwise. The reaction mixture was stirred for 2h before a saturated aqueous solution of ammonium chloride (50 ml) was added. The resulting mixture was extracted with ethyl acetate (3 x 100 ml); the organics were combined, washed with brine (100 ml), dried and evaporated. Purification by column chromatography (40 % [10 % Et_3N in EtOAC]/petrol) yielded the *alcohol* as an orange solid. Yield 63 %, ^1H NMR ($CDCl_3$, 300 MHz): δ 8.56 (d, 1H, J = 5.1 Hz), 8.48 (d, 1H, J = 5.1 Hz), 8.34 (m, 1H), 8.17 (m, 1H), 7.29 (m, 1H), 7.08 (m, 1H), 5.98 (ddd, J = 16.0, 10.0, 3.9 Hz, 1H), 5.34 (dt, J = 16.0, 1.3 Hz, 1H), 5.23 (br. d, J = 16.0 Hz, 1H), 5.17 (dt, J = 10.0, 1.3 Hz, 1H), 2.39 (s, 3H).

EXAMPLE 10



4'-(3-Chloro-propenyl)-4-methyl-[2,2']bipyridinyl. The alcohol from Example 9 (6.3 mmol, 1.5 g) was dissolved in dry dichloromethane (30 ml) and stirred at 0 °C before thionyl chloride (30 ml) was added in one portion. The reaction was stirred until tlc showed consumption of all starting material. The reaction mixture was allowed to warm to room temperature before careful addition of water (50 ml) and sodium bicarbonate (50 ml). The mixture was then extracted with dichloromethane (3 x 100 ml); the combined organics were dried over silica and then filtered through a plug of celite before being concentrated *in vacuo* to yield a yellow oil which was used without purification.

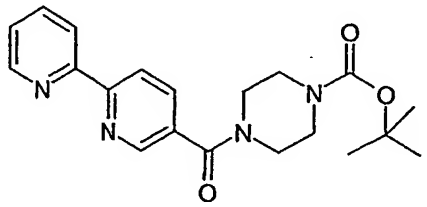
EXAMPLE 11



4-Methyl-4'-[3-(4-methyl-piperazin-1-yl)-propenyl]-2,2'-bipyridine. The chloride from Example10 (1.32 mmol, 0.342 g) was dissolved in dry dichloromethane (25 ml) at ambient

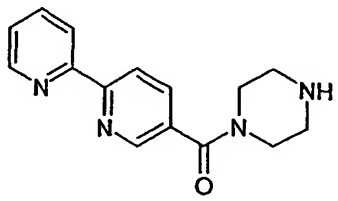
temperature. Piperazine (13.2 mmol, 1.13 g) was added and the solution was stirred overnight. The reaction mixture was extracted with hydrochloric acid (3 x 20 ml, 1M). The combined aqueous were washed with dichloromethane (10 ml), basified to pH 10 and extracted with dichloromethane (3 x 50 ml). The combined organics were washed with brine (50 ml) dried over sodium sulphate and then concentrated *in vacuo*. chromatography (10 % MeOH/DCM) yielded the *amine* as a mixture of geometric isomers. Yield 66 %, ^{1}H NMR ($CDCl_3$, 300 MHz) (major isomer reported): δ 8.53 (d, J = 5.1 Hz, 1H), 8.46 (d, J = 5.1 Hz, 1H), 8.31 (s, 1H), 8.16 (s, 1H), 7.18 (m, 1H), 7.06 (m, 1H), 6.65-6.48 (m, 2H), 3.14 (m, 2H), 2.60-2.40 (br. s, 8H), 2.38 (br. s, 3H), 2.24 (br. s, 3H).

EXAMPLE 12



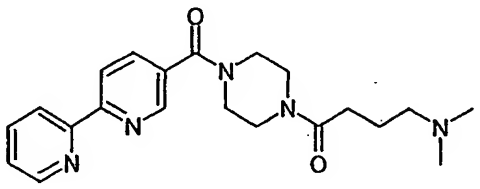
4-([2,2']Bipyridinyl-5-carbonyl)-piperazine-1-carboxylic acid tert-butyl ester. To a dry mixture of *N*-butoxycarbonyl piperazine (5 mmol, 1.1 g), 2,2'-bipyridyl-4-carboxylic acid (5 mmol, 1.0 g), EDC (6.5 mmol, 1.25 g) and hydroxybenzotriazole monohydrate (6.0 mmol, 0.81 g) was added dry dichloromethane (50 ml). The mixture was stirred at ambient temperature for 16 h before being washed with a saturated solution of sodium bicarbonate (10 ml), water (10 ml), brine (10 ml), dried over sodium sulphate and condensed *in vacuo*. The product was used without further manipulation. ^{1}H NMR ($CDCl_3$300 MHz) δ 8.72 (m, 2H), 8.50 (d, J = 8.0 Hz, 1H), 8.43 (d, J = 8.0 Hz, 1H), 7.88 (m, 2H), 7.36 (m, 1H), 3.90-3.25 (m, 8H), 1.52 (s, 9H).

EXAMPLE 13



[2,2']Bipyridinyl-5-yl-piperazin-1-yl-methanone. The product of example 12 (0.19mmol, 73 mg) was dissolved in dichloromethane (5 ml) at ambient temperature. Trifluoroacetic acid (1 ml) was added and stirring continued for 1 h. The reaction mixture was washed with water (2 x 10 ml) and the combined aqueous basified to pH 10 before being extracted with dichloromethane (2 x 10 ml). The combined organics were washed with brine, dried over sodium sulphate and concentrated in *vacuo* to give the amine. Yield: 47 %. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.72 (m, 2H), 8.48 (d, J = 8.1 Hz, 1H), 8.42 (d, J = 8.1 Hz, 1H), 7.85 (m, 2H), 7.35 (dd, J = 7.9, 4.9 Hz, 1H), 3.90-3.40 (br m, 4H), 3.50- 2.80 (br m, 4H).

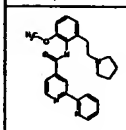
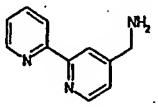
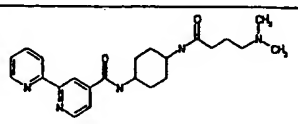
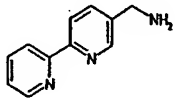
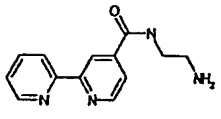
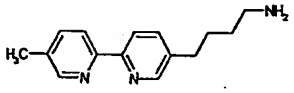
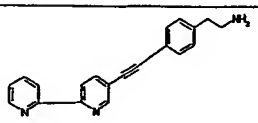
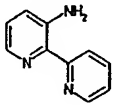
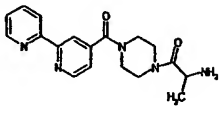
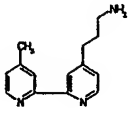
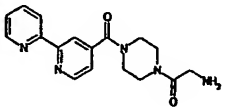
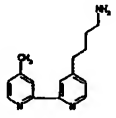
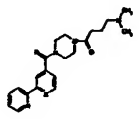
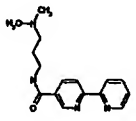
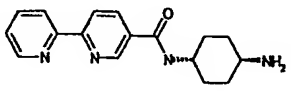
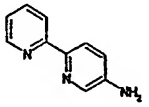
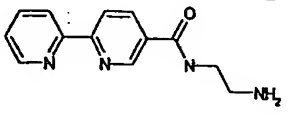
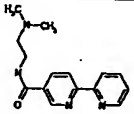
EXAMPLE 14

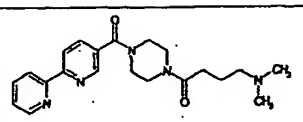
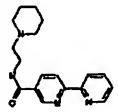
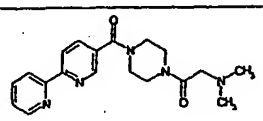
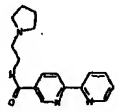
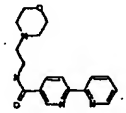
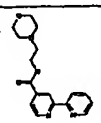
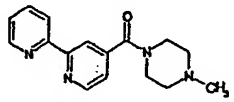
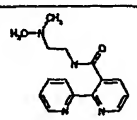
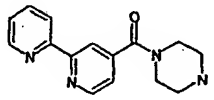
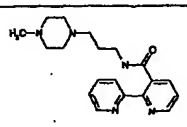
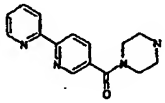
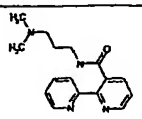
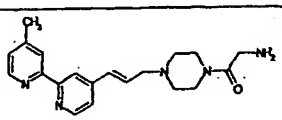
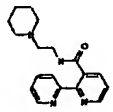
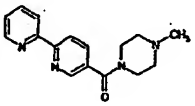
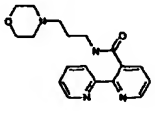
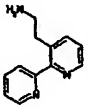
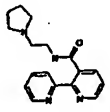
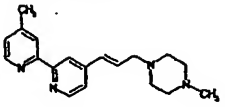
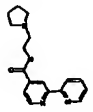


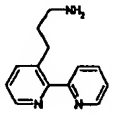
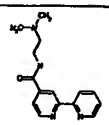
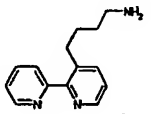
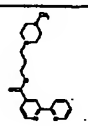
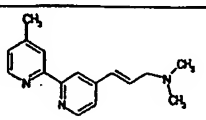
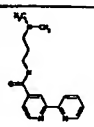
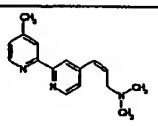
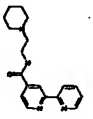
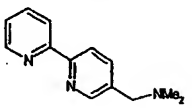
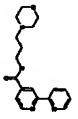
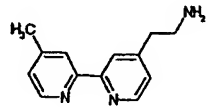
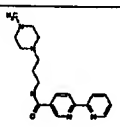
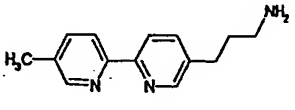
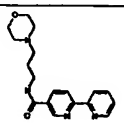
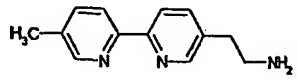
1-[4-([2,2']Bipyridinyl-5-carbonyl)-piperazin-1-yl]-4-dimethylamino-butan-1-one. A screw-top vial was charged with PS-carbodiimide resin (200mg) followed by a solution of γ-dimethylaminobutyric acid (0.15mmol, 25 mg) in dichloromethane (1 ml). The suspension was stirred gently for 5 min. before the addition of a solution of the amine from Example 13 (0.1mmol, 25 mg) in dichloromethane (1 ml). Stirring continued for 16 h before the addition of PS-trisamine (200 mg) and further stirring for 2 h. The solids were removed by filtration and the residue washed with dichloromethane (10 ml). The combined organics were dried in vacuo to give the tertiary amine. Yield: 41 mg (99 %). 1H NMR (CDCl3,300 MHz): δ 8.70 (m, 2H), 8.45 (d, J = 8.1 Hz, 1H), 8.40 (d, J = 8.1 Hz, 1H), 7.85 (m, 2H), 7.38 (dd, J = 4.9, 4.7 Hz, 1H), 3.95- 3.45 (m, 8H), 3.35 (t, J = 7.2 Hz, 2H), 2.80 (s, 6H), 2.68 (t, J = 6.5 Hz, 2H), 2.54 (app. q, J = 7.0 Hz, 2H).

In analogous manners 2,2´-bipyridyl amines are synthesised according to table 2, giving an representative example for a "C"-type library.

Table 2 2,2´-Bipyridyl amines in a "C"-type library.

Structure	Method	Structure	Method
	Ex 3		Ex 5
	Ex 14		Ex 5
	Ex 13		Ex 7
	Ex 7		Ex 4
	Ex 14		Ex 7
	Ex 14		Ex 7
	Ex 14		Ex 3
	Ex 13		Ex 4
	Ex 13		Ex 3

	Ex 14		Ex 3
	Ex 14		Ex 3
	Ex 3		Ex 3
	Ex 13		Ex 3
	Ex 13		Ex 3
	Ex 13		Ex 3
	Ex 14		Ex 3
	Ex12		Ex 3
	Ex 7		Ex 3
	Ex 11		Ex 3

	Ex 7		Ex 3
	Ex 7		Ex 3
	Ex 11		Ex 3
	Ex 11		Ex 3
	Ex 11		Ex 3
	Ex 7		Ex 3
	Ex 7		Ex 3
	Ex 7		

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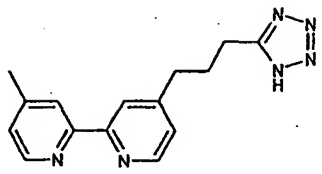
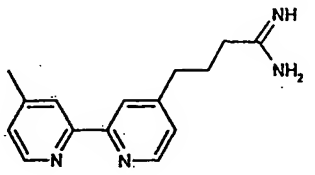
EXAMPLE 15



4'-Methyl-4-[3-(1H-tetrazol-5-yl)-propyl]-2,2'-bipyridine. 4-(3-Cyanopropyl)-4'-methyl-2,2'-bipyridyl (0.72 g, 3 mmol) was dissolved in dry toluene (10 ml), followed by addition of sodium azide (0.6 g, 9 mmol) and triethylammonium chloride (1.25 g, 9 mmol). The reaction was heated to 100 °C for 18 hours. After cooling, a small amount of water is added, the phases separated, and the aqueous phase acidified with hydrochloric acid. The crude product precipitated as a red oil, which is purified on a column (silica, EtOAc:MeOH 1:2). Yield: 0.6 g (71 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.13 (m, 2H), 2.41(s, 3H), 2.69 (t, J = 7.35 Hz, 2H), 2.92 (t, J = 7.45 Hz, 2H), 5.79 (s, broad, 1H)), 7.08 (m, 1H), 7.14 (m, 1H), 8.02 (s, 1H), 8.07 (s, 1H), 8.46 (d, J = 5.27 Hz, 1H), 8.48 (d, J = 5.09 Hz, 1H).

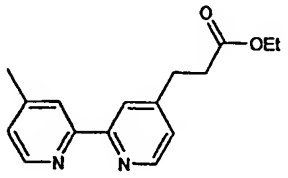
EXAMPLE 16



4-(4-Butyramidine)-4'-methyl-2,2'-bipyridine. Dry NH_4Cl (0.17g, 3mmol) in dry toluene (3 ml) was stirred at an ice-bath under nitrogen, and trimethylaluminium (1.6 ml, 2.0M, 3.2 mmol) added slowly. The mixture was allowed to attain room temperature. 4-(3-Cyanopropyl)-4'-methyl-2,2'-bipyridine (0.25 g, 1 mmol) was added, and the reaction is heated to 90 °C for 3 days. Alumina (9 g) was suspended in chloroform (40 ml), and the reaction mixture poured into it, followed by methanol (50 ml), and the reaction mixture was stirred for 0.5 hours. The slurry was filtered and concentrated *in vacuo*. Extractive work-up in DCM and aqueous $NaHCO_3$. Purification on alumina column (heptane:ethylacetate:ethanol (2:2:1)). Yield: 0.05 g (18 %). ^{1}H NMR (DMSO-d_6, 300

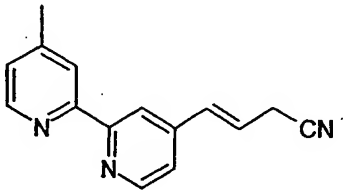
MHz): δ 1.85 (p, J = 7.7 Hz, 2H), 2.10 (t, J = 7.7 Hz, 2H), 2.42 (s, 3H), 2.68 (t, J = 7.7 Hz, 2H), 6.74 (br. s, 1H), 7.28 (m, 2H), 8.23 (m, 2H), 8.55 (m, 2H).

EXAMPLE 17



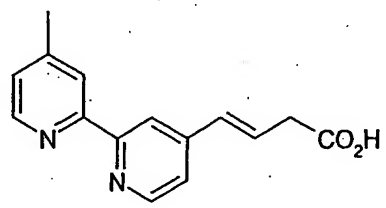
Ethyl 4-(3-carboxypropyl)-4'-metyl-2,2'-bipyridyl: 4,4'-Dimethyl-2,2'-bipyridyl (2.5 g, 13.5 mmol) was dissolved in dry THF (20 ml) under a nitrogen atmosphere, in a flame-dried flask, equipped with a stirrer. The solution was cooled to –78 °C, and a solution of LDA (10 ml, 16.8 mmol) was added. The reaction mixture was allowed to warm to room temperature for 1,5 hours. This solution was cannulated into a solution of ethyl 2-bromoacetate (2.3 ml, 20 mmol) in dry THF (15 ml) at –78 °C, placed in a flame-dried flask under a nitrogen atmosphere, equipped with a stirrer. The reaction mixture was allowed to reach room temperature slowly over night, and quenched by addition of a saturated aqueous solution of sodium bicarbonate. Extractive work-up using ethyl acetate, drying and evaporation, gave the crude product. Purified by column chromatography (Silica; DCM:MeOH:NH_4OH 95:5:0.5). Yield: 1.86 g (51 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.17 (t, J = 7.16 Hz, 3H), 2.40 (s, 3H), 2.66 (t, J = 7.63 Hz, 2H), 2.99 (t, J = 7.63 Hz, 2H), 4.07 (q, J = 7.16 Hz, 2H), 7.13 (m, 2H), 8.22 (s, 1H), 8.28 (s, 1H), 8.49 (d, J = 5.08 Hz), 8.52 (d, J = 5.09 Hz, 1H).

EXAMPLE 18



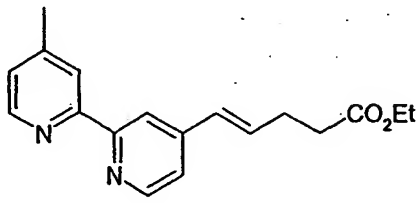
4-(4'-Methyl-[2,2']bipyridinyl-4-yl)-but-3-enenitrile. The chloride from Example 10 (3.5 mmol, 0.868g) was dissolved in dry ethanol (40 ml) at ambient temperature. Potassium cyanide (4.3 mmol, 0.209g) dissolved in water (3.5 ml) was added in one portion and the resulting solution was heated to 78 °C for 15 h. The solvent was then removed *in vacuo* before the crude product was subjected to column chromatography (40 % [10 % Et_3N in EtOAC]/petrol) yielded the cyanide as an orange solid. Yield: 18 %, ^1H NMR ($CDCl_3$, 300 MHz): δ 8.65 (d, J = 5.1 Hz, 1H), 8.55 (d, J = 5.1 Hz, 1H), 8.43 (s, 1H), 8.24 (s, 1H), 7.29 (dd, J = 4.9, 0.8 Hz, 1H), 7.16 (dd, J = 4.9, 0.8 Hz, 1H), 6.80-6.55 (m, 2H), 3.78 (d, J = 7.2 Hz, 2H), 2.45 (s, 3H).

EXAMPLE 19



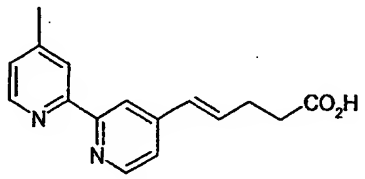
4-(4'-Methyl-[2,2']bipyridinyl-4-yl)-but-3-enoic acid. The cyanide from Example 18 (0.48 mmol, 0.112g) was taken up in sodium hydroxide solution (20 mmol, 4M, 5 ml) and stirred for 15h at reflux. The solvent was then remove *in vacuo* and the product purified on chromatotron (EtOAc,/petrol gradient). Yield 3.2 %, ^1H NMR ($CDCl_3$, 300 MHz): δ 8.62 (d, J = 5.1 Hz, 1H), 8.55 (d, J = 5.1 Hz, 1H), 8.39 (s, 1H), 8.25 (s, 1H), 7.27 (m, 1H), 7.16 (m, 2H), 6.71 (m, 1H), 4.41 (m, 2H), 2.46 (s, 3H).

EXAMPLE 20



5-(4'-Methyl-[2,2']bipyridinyl-4-yl)-pent-4-enoic acid ethyl ester. The alcohol from Example 9 (0.64 mmol, 0.144g) was dissolved in triethyl orthoacetate (2 ml), and toluene (8 ml) under a nitrogen atmosphere. Acetic acid (20 µl) was added and the resulting solution was heated to 120 °C for 3 h. After cooling to ambient temperature, a saturated solution of sodium carbonate (10 ml) was added and the mixture extracted with ethyl acetate (3 x 20 ml). The combined organics were washed with brine (50 ml) before the addition of petrol (120 ml). The organic solution was passed through a plug of silica and the filtrate reduced *in vacuo*. Purification by column chromatography (20 % [10 % Et_3N in EtOAC]/petrol) yielded the *ester* as an pale yellow solid. Yield: 40 %.

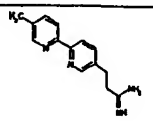
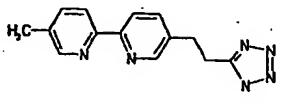
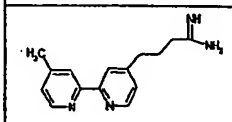
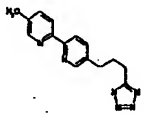
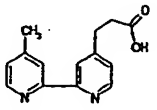
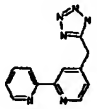
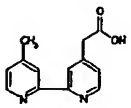
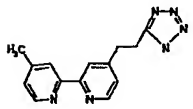
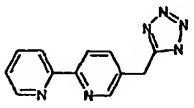
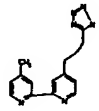
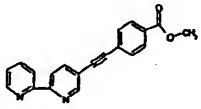
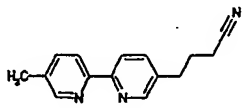
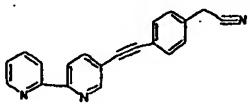
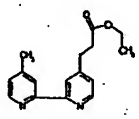
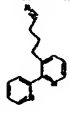
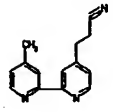
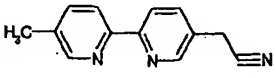
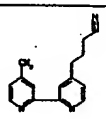
EXAMPLE 21

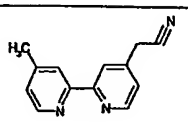
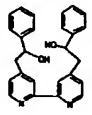
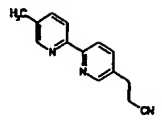
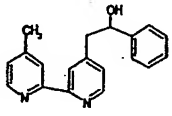


5-(4'-Methyl-[2,2']bipyridinyl-4-yl)-pent-4-enoic acid. Ethyl ester from Example 20 (0.045 mmol, 0.020g) was taken up in a mixture of THF (0.5 ml), ethanol (0.5 ml) and water (0.1 ml) at ambient temperature before potassium carbonate (0.045 mmol, 0.063 g) was added and the resulting suspension stirred overnight. Barium hydroxide (0.1 g) was added and the suspension stirred for a further 7 h before the pH of the mixture was adjusted to 5 and the mixture extracted with ethyl acetate (3 x 5 ml). The combined organics were dried and reduced *in vacuo*. Yield: 50 %, ^1H NMR ($CDCl_3$, 300 MHz) δ 8.60 (m, 2H), 8.33 (s, 1H), 8.24 (s, 1H), 7.21 (dd, J = 6.1, 4.9 Hz, 1H), 6.66 (m, 1H), 6.51 (d, J = 15.8 Hz, 1H), 2.62 (m, 4H), 2.46 (s, 3H).

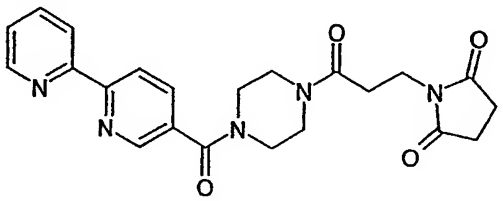
In analogous manners the following functional groups were introduced to the 2,2´-bipyridine scaffold, shown in table 3, giving an representative example for various kinds of "C"-type libraries.

Table 3 Various functionalized 2,2'-bipyridines as part of various "C"-type libraries.

Structure	Method	Structure	Method
	Ex 16		Ex 15
	Ex 16		Ex 15
	Ex 21		Ex 15
	Ex 21		Ex 15
	Ex 15		Ex 15
	Ex 8		Ex 6
	Ex 8		Ex 17
	Ex 6		Ex 6
	Ex 6		Ex 6

	Ex 6		Ex 17
	Ex 6		Ex 17

EXAMPLE 22



1-{3-[4-([2,2']Bipyridinyl-5-carbonyl)-piperazin-1-yl]-3-oxo-propyl}-pyrrolidine-2,5-dione.

The corresponding maleimide (made analogously to Example 14) (0.11mmol, 43 mg) was dissolved in methanol (5 ml) at ambient temperature before 10 % palladium on carbon (10 mg) was added and the atmosphere exchanged first with nitrogen and second with hydrogen. The suspension was stirred vigorously for 16 h and then the reaction mixture was filtered through a plug of celite. The residue was washed with methanol (50 ml). The combined organics were evaporated to dryness to give the succinimide. Yield: 99 %. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.72 (m, 2H), 8.46 (d, J = 8.1 Hz, 1H), 8.39 (d, J = 8.1 Hz, 1H), 7.87 (m, 2H), 7.36 (m, 1H), 3.90-3.40 (m, 10H), 2.70 (m, 6H).

By the same procedure the following compound was made from the unsaturated amide:

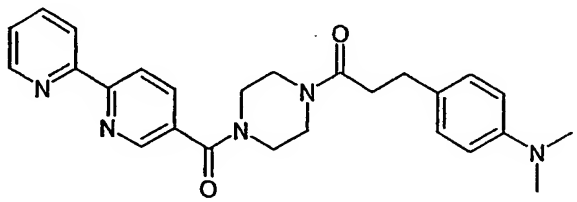
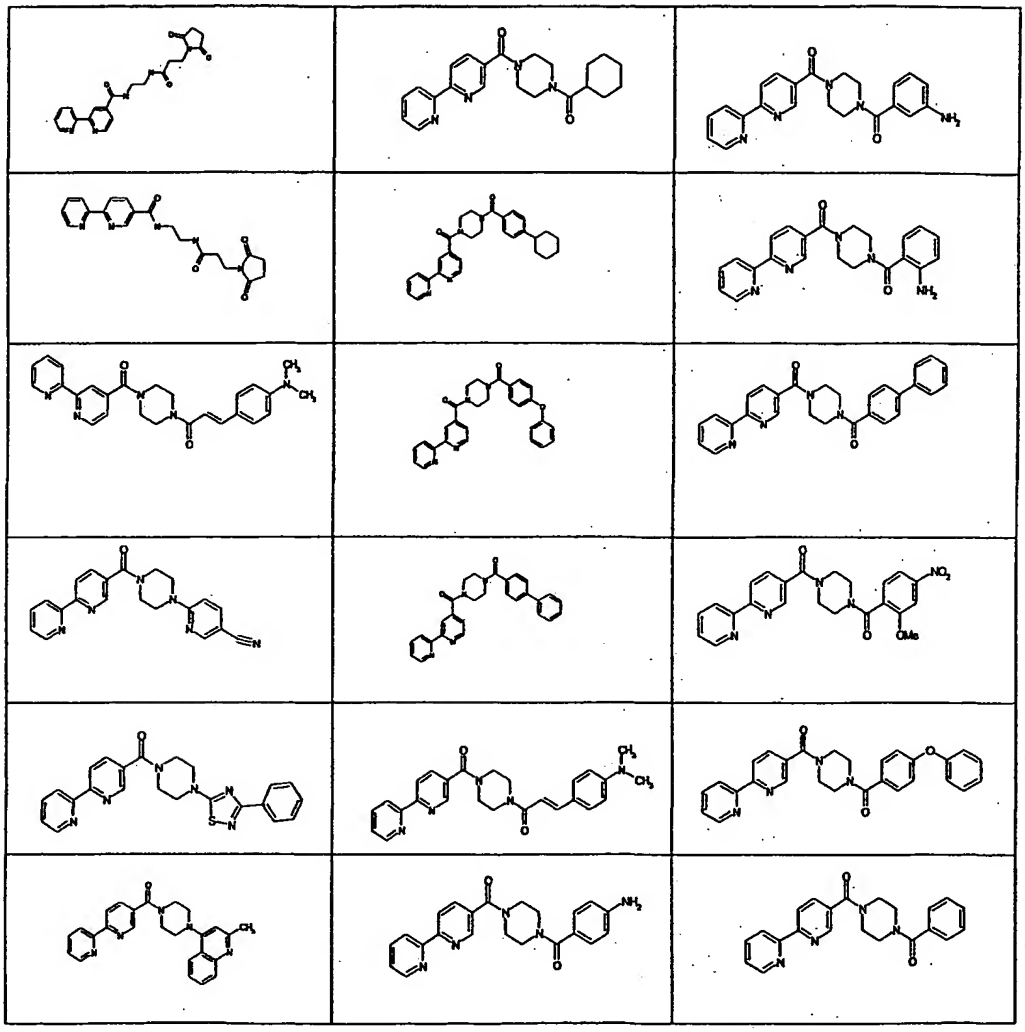
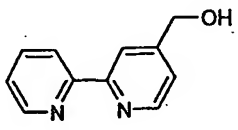


Table 4 2,2'-Bipyridines with various C-groups in a typical "A"-type library made similarly to Example 14.

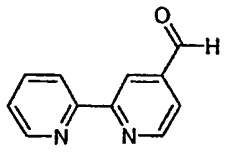


EXAMPLE 23



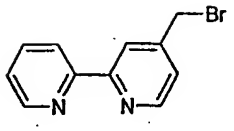
4-(Hydroxymethyl)-2,2′-bipyridine. Methyl 2,2′-bipyridine-4-carboxylate (9.34 mmol, 2.0 g) was dissolved in MeOH/DCM (5 ml/50 ml), whereupon $LiBH_4$ (18.67 mmol, 0.4 g, 2 equiv.) was added and the reaction mixture was stirred at room temperature for 3h. Another portion of $LiBH_4$ (9.33 mmol, 0.2 g, 1 equiv.) was added and the reaction mixture was stirred at room temperature for 16 h. The reaction was quenched with acetone. The solvent was removed *in vacuo* after which the solid residue was dissolved in DCM, and chromatographed on a silica column (DCM/MeOH/NH_3, 100/10/1).Yield: 92 %. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.59 (ddd, J= 0.93, 1.5, 3.96 Hz, 1H), 8.52 (dd, J= 0.57, 2.25 Hz, 1H), 8.29 (dt, J= 0.96, 8.07 Hz, 1H), 8.26-8.25 (m, 1H), 7.77 (td, J= 1.86, 7.80 Hz, 1H), 7.29-7.23 (m, 2H), 4.71 (s, 2H), 4.28 (br. s, 1H).

EXAMPLE 24



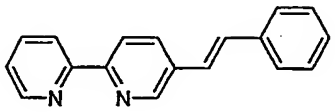
4-(Carboxaldehyde)-2,2′-bipyridine. Oxalyl chloride (23.64 mmol, 2.1ml, 1.5 equiv.) was dissolved in dry DCM (30 ml) and cooled to –78 °C. DMSO (31.5 mmol, 2.2 ml, 2 equiv.) was dissolved in DCM (15 ml) and was thereafter added dropwise to the oxalyl chloride solution. The 4-(methylhydroxy)-2,2′-bipyridine (15.76 mmol, 2.0g) dissolved in DCM (15 ml) was then added, and the mixture was stirred at -78 °C for 5 h under an N_2-atmosphere. Triethyl amine (78.8 mmol, 11.0 ml, 5 equiv.) was then added and the reaction mixture was allowed to warm to ambient temperature. DCM (100 ml) was added and sat. $NaHCO_3$ (150 ml) was added. The organic phase was separated and the aqueous phase was extracted with DCM (2x100 ml). The combined organic phases were dried over $MgSO_4$, and the solvent was evaporated *in vacuo* The crude product was purified by column chromatography (DCM/MeOH/NH_3, 100/10/1). Yield: 44 %. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.18 (s, 1H), 8.90-8.83 (m, 1H), 8.73-8.70 (m, 2H), 8.50-8.39 (m, 1H), 7.86 (td, J= 1.71, 7.5 Hz, 1H), 7.73 (dd, J= 1.53, 4.95 Hz, 1H), 7.39-7.34 (m, 1H).

EXAMPLE 25



4-(Bromomethyl)-2,2´-bipyridine. 4-(Hydroxymethyl)-2,2´-bipyridine (5.37 mmol, 1.0 g) was dissolved in DMF (15 ml). PBr_3 (5.37 mmol, 0.5 ml) was added dropwise at room temperature under inert atmosphere. The reaction mixture was stirred at room temperature for 15 h. Water (50 ml) was added as the reaction vessel was cooled on an ice-bath. Ethyl acetate (100 ml) was added, and sat. $NaHCO_3$ (100 ml) was added. The organic layer was separated and the aqueous phase was extracted with ethyl acetate (2x50 ml). The combined organic layers were dried over $MgSO_4$, and the solvent was evaporated *in vacuo*. Column chromatography of the crude material (DCM/MeOH, 100/10) yielded the pure bromo methyl compound. Yield. 60 %. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.69-8.66 (m, 2H), 8.44-8.41 (m, 2H), 7.84 (td, J= 1.86, 7.5 Hz, 1H), 7.36-7.31 (m, 2H), 4.49 (s, 2H).

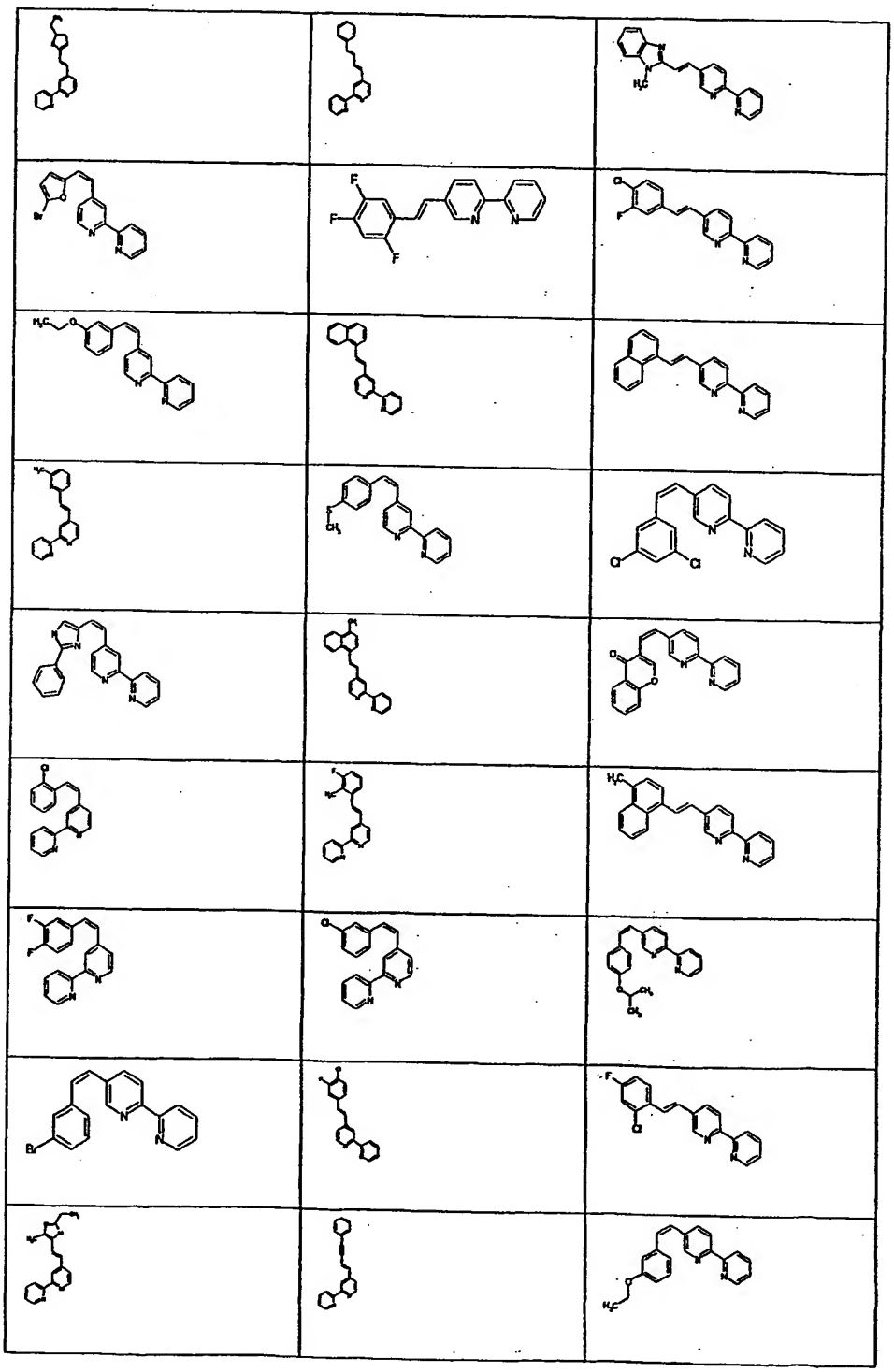
EXAMPLE 26

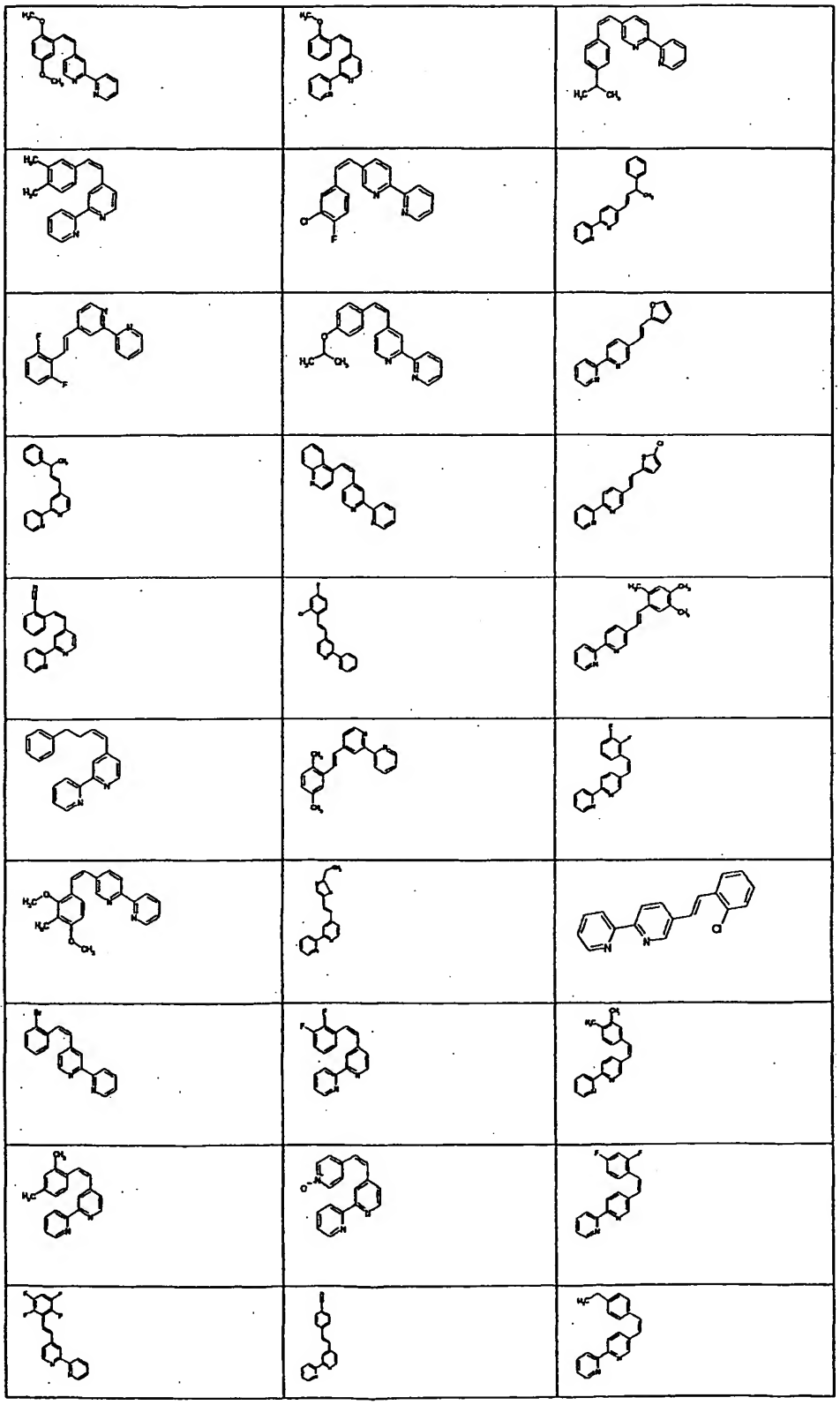


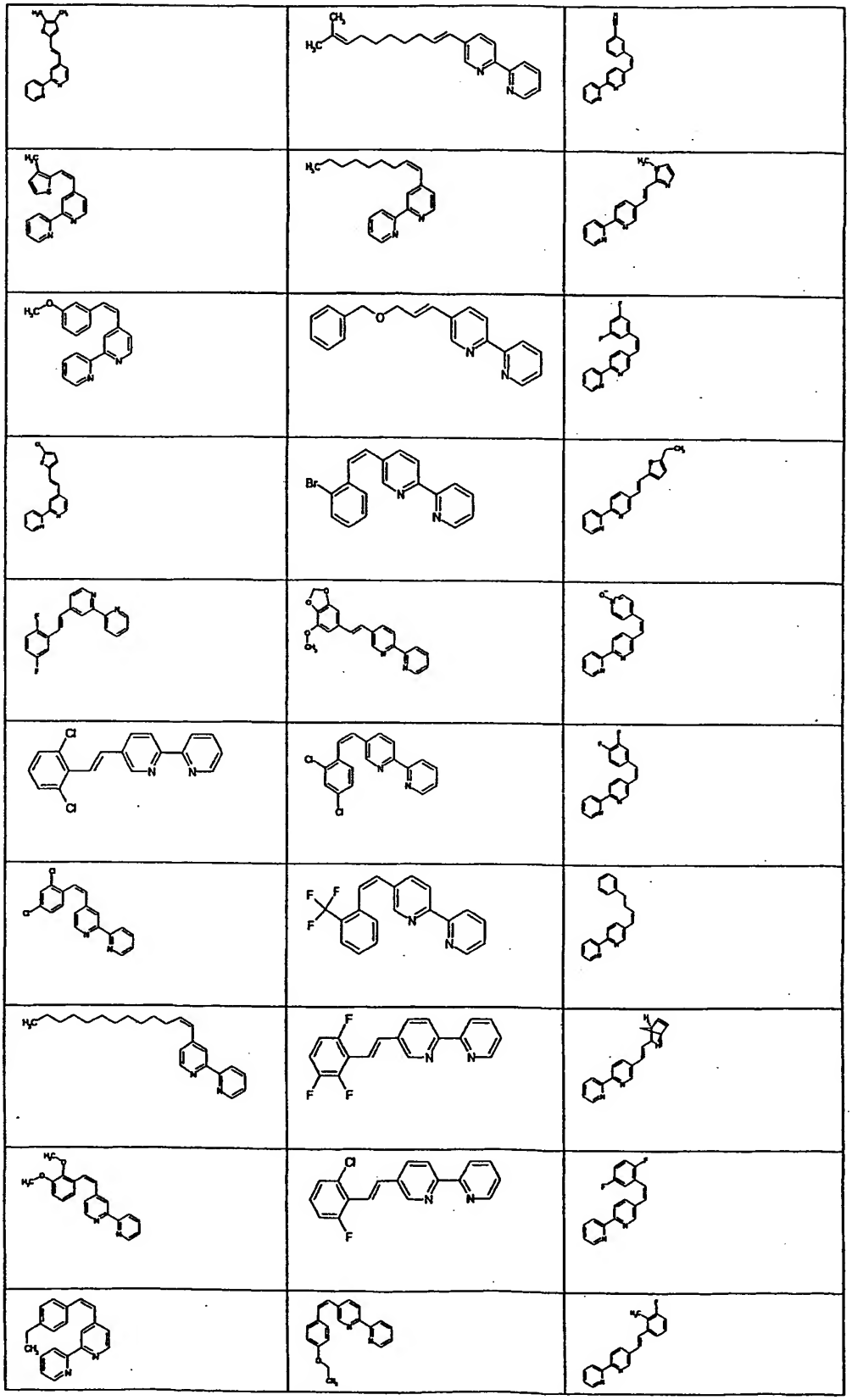
5-(2-Phenyl-1-ethenyll)-2,2´-bipyridine. Triphenyl phosphine (1.0 mmol, 0.262 g) was dissolved in dry benzene (7 ml). 4-(Bromomethyl)-2,2´-bipyridine (1.06 mmol, 0.265 g) was added, and the reaction solution was refluxed for 2 h. A white precipitate formed. The solvent was removed *in vacuo*, and DCM (7 ml) was added to the solid residue. Benzaldehyde (1.0 mmol, 0.102 ml) was added and thereafter aqueous NaOH (1.0 mmol, 0.25 ml, 4M). The reaction mixture was stirred at room temperature for 15 h. Addition of $MgSO_4$ to remove water was followed by filtration through a short silica column to yield a clear colourless solution. Purification by column chromatography (10 % EtOH/DCM) yielded the desired pure product. Yield: 83 %. ^{1}H NMR ($CDCl_3$, 300 MHz) (selected peaks): δ 6.84 (d, J = 12.1 Hz, 1H), 6.62 (d, J = 12.3 Hz, 1H).

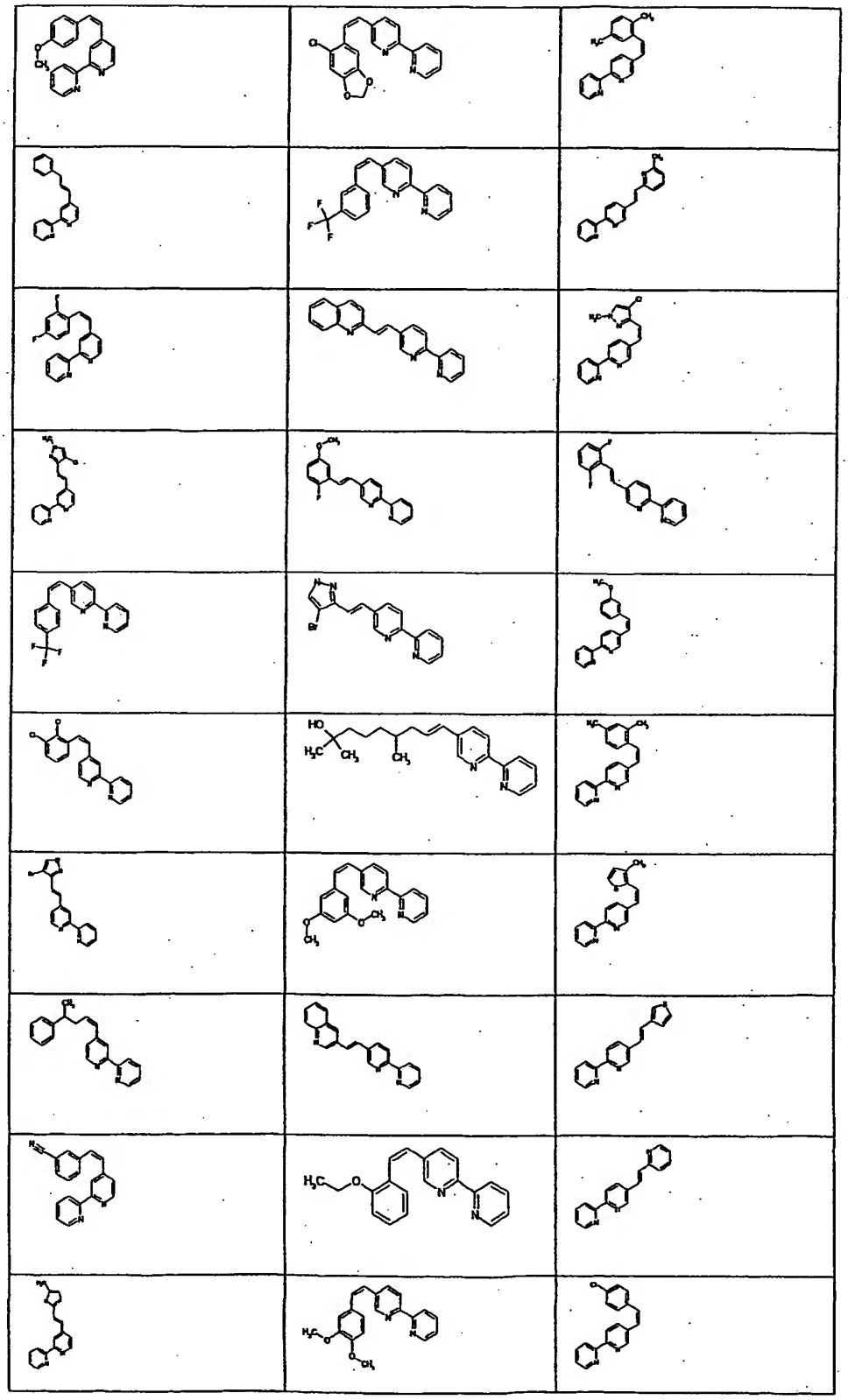
In analogous manners, bipyridyl alkenes have been synthesized according to Table 5.

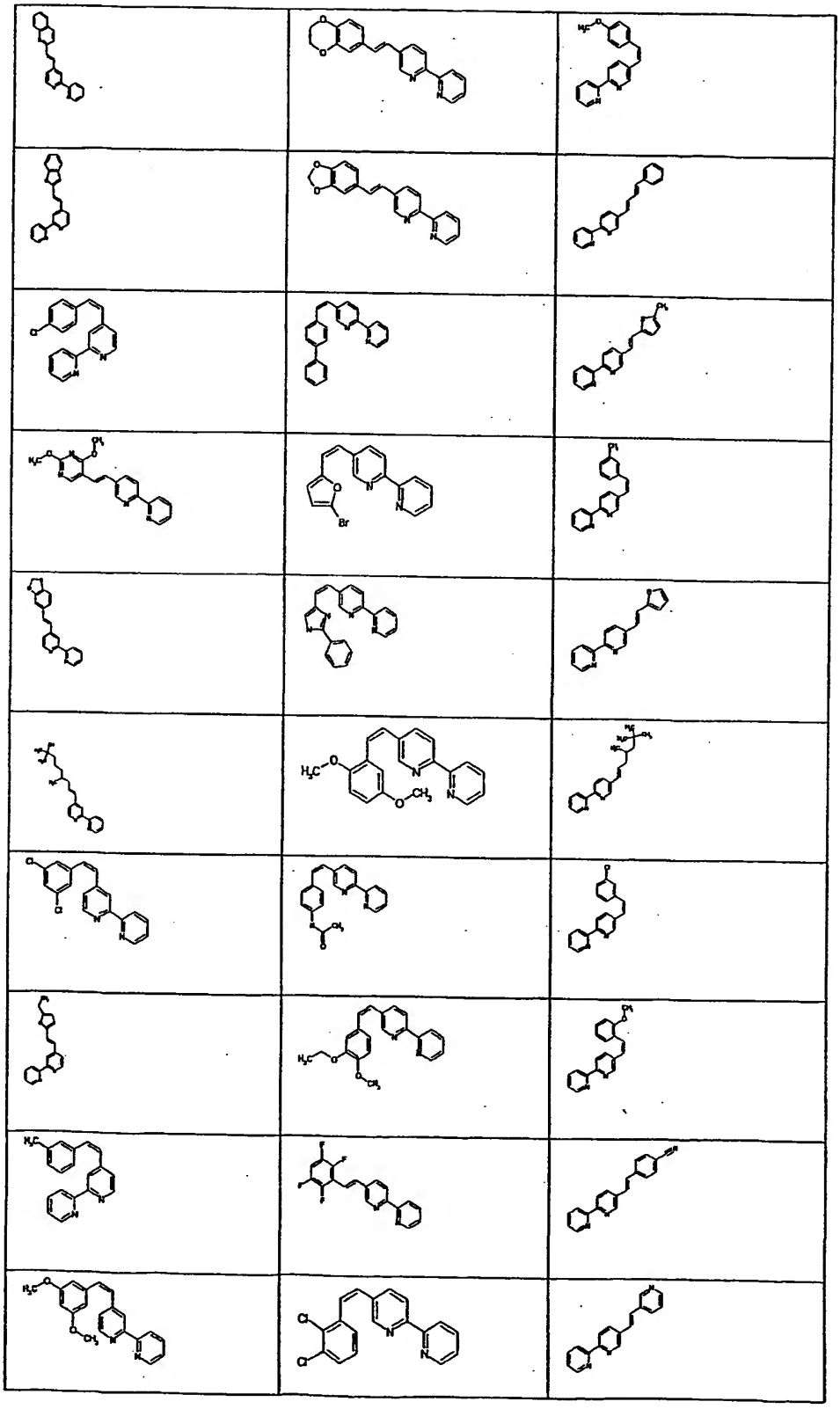
Table 5 Alkene derivatives prepared according to Example 26 in a typical "A"-type library.

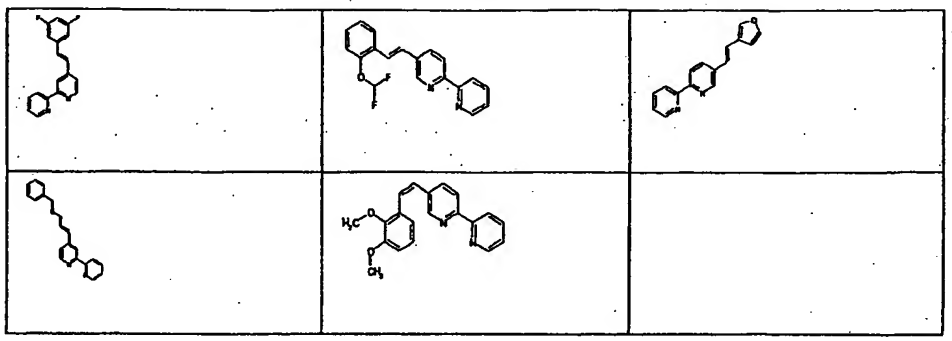




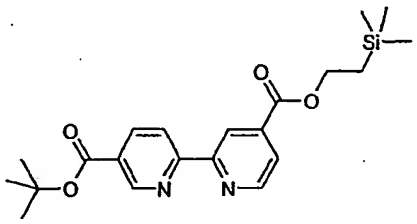








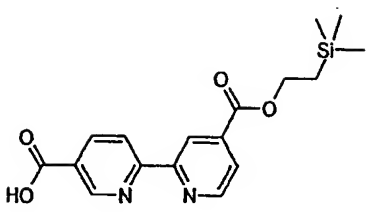
EXAMPLE 27



4-(2-(trimethylsilyl)-ethylcarboxylate)-5'-(tert-butylcarboxylate)-2,2'-bipyridine. 6-Chloro-*tert*-butylnicotinate (12.7 mmol, 2.7g) was dissolved in dry *m*-xylene (150 ml) whereupon $Me_3SnSnMe_3$ (15.26 mmol, 5.0 g) was added together with $PdCl_2(PPh_3)_2$ (1.5 mmol, 1.0g). The reaction solution was heated to 130C under an N_2 atmosphere for 4h. 2-Chloro-(2-(trimethylsilyl)ethyl)-*iso*-nicotinate (15.26 mmol, 3.9g) was added and stirring was continued at 130C for 16h. The reaction mixture was allowed to cool to ambient temperature whereafter the solvent was evaporated *in vacuo*. The residue was taken up in DCM, and purified by column chromatography using DCM as the eluent. Yield: 62 %. ^1H NMR ($CDCl_3$, 300 MHz): δ 9.29-9.25 (m, 1H), 9.05-9.02 (m, 1H), 8.88-8.84 (m, 1H), 8.56-8.51 (m, 1H), 8.42-8.36 (m, 1H), 7.96-7.92 (m, 1H), 4.53-4.48 (m, 2H), 1.65 (s, 9H), 1.24-1.18 (m, 2H), 0.12 (s, 9H).

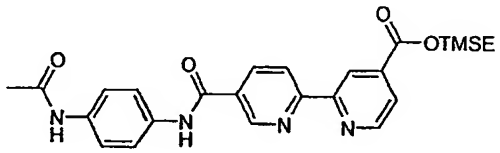
By the same method the corresponding 2,2'-bipyridyl-esters, -nitriles, -aldehydes, -protected amines and protected alcohols be synthesised in all possible combinations, and positions on the 2,2'-bipyridyl scaffold (*i.e.* AA'-, AC-libraries).

EXAMPLE 28



4-(2-(trimethylsilyl)-ethylcarboxylate)-5′-(carboxyacid)-2,2′-bipyridine. 4-(2-(Trimethylsilyl)-ethylcarboxylate)-5′-(*tert*-butylcarboxylate)-2,2′-bipyridine (2.5 mmol, 1.0 g) was dissolved in dry 1,4-dioxane (15 ml). Triethylamine (3.75 mmol, 0.523 ml) was added and TMSOTf (3.75 mmol, 0.679 ml) was added droppwise. Upon completion of addition the reaction solution was heated to 100C for 2h. Stirring was thereafter continued for 3h at 21C. Water was then carefully added and the formed precipitate was collected by filtration and the solid residue was washed several times with water and allowed to dry at room temperature for 24h. Yield: 71 %. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 9.21-9.18 (m, 1H), 8.94-8.90 (m, 1H), 8.87-8.83 (m, 1H), 8.54-8.52 (m, 1H), 8.45-8.42 (m, 1H), 7.93-7.90 (m, 1H), 4.49-4.43 (m, 2H), 1.18-1.15 (m, 2H), 0.08 (s, 9H).

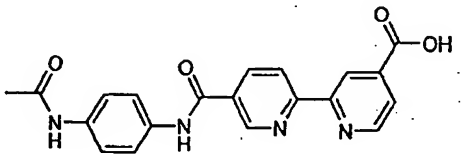
EXAMPLE 29



4-(2-(trimethylsilyl)-ethylcarboxylate)-5′-(4-(acetanilido)carboxamide)-2,2′-bipyridine. 4-(2-(Trimethylsilyl)-ethylcarboxylate)-5′-(carboxyacid)-2,2′-bipyridine (1.45 mmol, 0.5g) was dissolved in DCM/DMF (5 ml / 5 ml). HBTU (1.74 mmol, 0.66g) was added and the mixture was stirred for 2h at room temperature. 4-Aminoacetanilide (1.74 mmol, 0.26g) was added in one portion, and stirring was continued at room temperature for another 16h. Water was added, and the reaction mixture was extracted with DCM. The combined organic phases were washed once with water and finally with brine prior to drying over $MgSO_4$, and evaporation *in vacuo*. Purification was made by column chromatography on neutral alumina using DCM/ ethanol (95:5) as eluent. Yield: 72 %. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.48 (s, 1H), 9.94 (s, 1H), 9.25-9.22 (m, 1H), 8.98-8.93 (m, 1H), 8.90-8.88

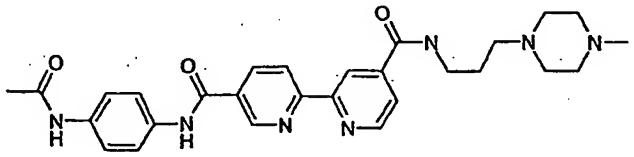
(m, 1H), 8.57-8.54 (m, 1H), 8.50-8.46 (m, 1H), 7.94-7.92 (m, 1H), 7.70 (d, *J* = 8.85 Hz, 2H), 7.58 (d, *J* = 9.03 Hz, 2H), 4.51-4.46 (m, 2H), 2.04 (s, 3H), 1.19-1.14 (m, 2H), 0.09 (s, 9H).

EXAMPLE 30



4-(carboxyacid)-5´-(4-(acetanilido)carboxamide)-2,2´-bipyridine. 4-(2-(Trimethylsilyl)-ethylcarboxylate)-5´-(4-(acetanilido)-carboxamide)-2,2´-bipyridine (0.042 mmol, 20 mg) was dissolved in THF (5 ml). To the solution was added TBAF (0.126 mmol, 126□l, 1M solution in THF). The solution was stirred at room temperature for 15h, whereupon it was made acidic with diluted HCl (1M solution) to pH = 3.5. The formed precipitate was collected by filtration and rinsed with several portions of water, and thereafter dried at room temperature for 24h. Yield: 67 %. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 13.80 (br. s, 1H), 10.47 (s, 1H), 9.92 (s, 1H), 9.24-9.23 (m, 1H), 8.95-8.84 (m, 2H), 8.57-8.54 (m, 1H), 8.48 (dd, *J* = 2.07, 7.95 Hz, 1H), 7.93 (dd, *J* = 1.68, 7.80 Hz, 1H), 7.70 (d, *J* = 8.46 Hz, 2H), 7.57 (d, *J* = 9.03 Hz, 2H), 2.04 (s, 3H).

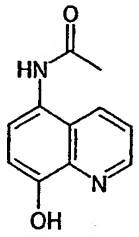
EXAMPLE 31



4-(3´´-(N-methylpiperazine)-propyl)carboxamide)-5´-(4-(acetanilido)carboxamide)-2,2´-bipyridine. 4-(Carboxyacid)-5´-(4-(acetanilido)carboxamide)-2,2´-bipyridine (0.04 mmol, 15 mg) was dissolved in DCM/DMF (1:1, 5 ml), whereupon HBTU (0.05 mmol , 189 mg) was

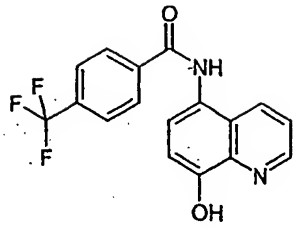
added in one portion. A few drops of triethylamine was added and the resulting mixture was stirred at room temperature for 3 h. 3-(*N*'-Methylpiperazine)-propyl amine (0.06 mmol , 9.4 mg) was added, and the reaction solution was stirred at room temperature over night. Water was added and the the organic layer was separated. The aqueous phase was extracted with DCM. The combined organic layers were washed with water, brine and finally sat. $CaCl_2$. Drying over $MgSO_4$, and evaporation *in vacuo* yielded a yellow viscous oil. Purification was made by column chromatography on neutral alumina using DCM/ ethanol (95:5) as eluent. Yield: 63 %. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.38 (s, 1H), 9.81 (s, 1H), 9.21-9.19 (m, 1H), 8.91-8.82 (m, 2H), 8.58-8.55 (m, 1H), 8.46 (dd, J = 1.97, 7.89 Hz, 1H), 7.90 (dd, J = 1.73, 7.81 Hz, 1H), 7.69 (d, J = 8.51 Hz, 2H), 7.53 (d, J = 9.10 Hz, 2H), 3.45-3.30 (m, 2H), 2.60-2.30 (m, 10H), 2.18 (s, 3H), 2.04 (s, 3H), 1.18-1.16 (m, 2H).

EXAMPLE 32



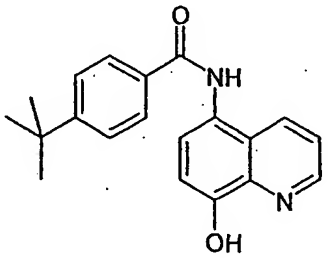
N-(8-Hydroxy-quinolin-5-yl)-acetamide. 5-Amino-8-hydroxyquinoline (1 mmol, 0.233g) was stirred in ether at ambient temperature before acetic anhydride (10 mmol, 1ml), followed by sodium acetate (10 mmol, 1.36g) was added. The resulting mixture was heated to 40 °C for 16 h before being diluted with ether (100ml) poured onto a saturated solution of ammonium chloride (50 ml). The organics were separated and washed with sodium bicarbonate (50 ml), water (3 x 50 ml), brine (50 ml), dried over sodium sulphate and concentrated *in vacuo*. Purification by column chromatography (30 % EtOAc/petrol). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.11 (dd, J = 8.9, 1.7 Hz, 1H), 9.4 (dd, J = 4.0, 1.5 Hz, 1H), 8.46 (d, J = 8.4 Hz, 1H), 7.71 (dd, J = 8.8, 4.1 Hz, 1H), 7.57 (d, J = 8.8 Hz, 1H), 2.56 (s, 3H).

EXAMPLE 33



N-(8-Hydroxy-quinolin-5-yl)-4-trifluoromethyl-benzamide. 5-Amino-8-hydroxyquinoline (0.15mmol, 25 mg) was dissolved in dry dichloromethane (5 ml) before the sequential addition of dimethyl formamide (0.2 ml), *N,N,*-dimethylaminopyridine (1 crystal), PS-carbodiimide (750mg) and 1-hydroxybenzotriazole monohydrate (0.6mmol, 81 mg). The suspension was stirred for 72 h before the solids were removed by filtration and the resulting filtrate diluted with dichloromethane (20 ml), washed with sodium bicarbonate (2 x 20 ml), brine (20 ml), dried over sodium sulphate and concentrated *in vacuo*. The residue was then taken up in dichloromethane (50 ml), with methanol (10 ml) and water (1 ml). To this mixture was added lithium hydroxide (30mmol, 720 mg). The suspension was stirred for 16 h before the solids were removed by filtration and the resulting filtrate washed with sodium bicarbonate (20 ml), water (20 ml) and brine (20 ml), dried over sodium sulphate and purified by direct filtration through a plug of alumina. The alumina was washed with dichloromethane (100 ml) before the product was eluted with ethyl acetate. The volatiles were removed *in vacuo*. GC-MS: m/z = 332 (= M^+).

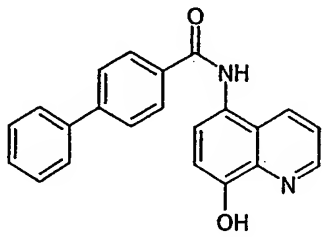
EXAMPLE 34



4-tert-Butyl-N-(8-hydroxy-quinolin-5-yl)-benzamide. To a suspension of 5-amino-8.hydroxyquinoline dihydrochloride (1.0 mmol, 0.23 g) and dimethylaminopyridine (3 crystals) in dichloromethane (10 ml) at ambient temperature was added 4-*tert*-butylbenzoyl chloride (3.0 mmol, 0.59 ml). Stirring continued for 10 min before triethylamine (10mmol, 2.8 ml) was added in one portion. The solution was allowed to stir overnight before all volatiles were removed *in vacuo* and the residue purified directly by

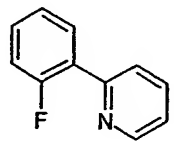
column chromatography (10 % EtOAc / hexane). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.13 (dd, J = 9.0, 1.7 Hz, 1H), 8.99 (dd, J = 4.1, 1.7 Hz, 1H), 8.52 (d, J = 8.5 Hz, 1H), 8.27 (dt, J = 8.5, 1.7 Hz, 2H), 7.69 (m, 2H), 7.61 (m, 2H), 1.43 (s, 9H).

In similar fashion the following compound was made:



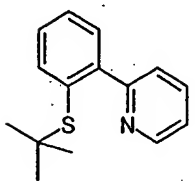
^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.14 (dd, J = 8.9, 1.5 Hz, 1H), 9.03 (dd, J = 4.0, 1.4 Hz, 1H), 8.57 (d, J = 8.5 Hz, 1H), 8.43 (app d, J = 8.1 Hz, 2H), 7.84 (app. d, J = 8.3 Hz, 2H), 7.70 (m, 4H), 7.50 (m, 4H).

EXAMPLE 35



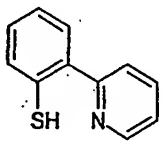
2-(2-Pyridyl)fluorobenzene: 2-Fluorophenylboronic acid (3.0 g, 21.4 mmol) was dissolved in DME (40 ml). 2-Bromopyridine (1.64 ml, 17.2 mmol) was added followed by 2M K_2CO_3 (20 ml). The mixture was degassed by bubbling nitrogen gas through for 34 min. Bis-(triphenylphosphine)palladium chloride (1.2 g, 1.72 mmol) was added and the mixture was heated to 80°C over night. The mixture was cooled to room temperature and filtered through celite. Extraction with H_2O (200 ml) and EtOAc (200 ml), drying the organic phase over $MgSO_4$, filter and evaporation gave the crude product. Purification by column chromatography (SiO_2, DCM: 10 % NH_4OH in MeOH 10:0.05). Yield: 2.4 g (80 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.18 (dt, J = 8.1, 1.13 Hz, 1H), 7.28 (m, 2H), 7.40 (m, 1H), 7.79 (m, 2H), 8.00 (dt, J = 7.72, 1.88 Hz, 1H), 8.75 (dt, J = 4.52, 1.32 Hz, 1H). LC-MS: m/z = 174 (=M+1)

EXAMPLE 36



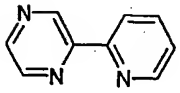
S-tert-Butyl-2-(2-pyridyl)thiophenol: DMF (10 ml) was degassed for 1hour and 10 minutes. Sodium hydride (60 % dispersion in mineral oil) (231 mg, 5.77 mmol) and 2-methyl-2-propanethiol (715 µl, 5.77 mmol) was added. The mixture was stirred for 7 minutes at room temperature. 2-(2-Pyridyl)fluorobenzene (500 mg, 2.89 mmol) was added, and the mixture was heated to 120 °C for 3 days. The mixture was cooled to room temperature. H_2O (50 ml) was added and the mixture was extracted with EtOAc (70 ml). The organic phase was washed with H_2O (50 ml), dried over $MgSO_4$, filtered and evaporated. Yield: ~100 %, ^1H NMR ($CDCl_3$, 300 MHz) δ 1.04 (s, 9H), 7.25 (m, 1H), 7.39 (dt, J = 7.54, 1.51 Hz, 1H), 7.49 (dt, J = 7.54, 1.51 Hz, 1H), 7.69 (m, 4H), 8.70 (m, 1H). LC-MS: m/z = 244 (=M+1)

EXAMPLE 37



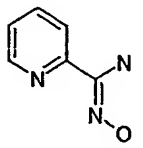
2-(2-Pyridyl)thiophenol: S-*tert*-Butyl-2-(2-pyridyl)thiophenol (200 mg, 0.82 mmol) was dissolved in 37 % HCl (4 ml) and the mixture was heated to 110 °C over night. The mixture was cooled to room temperature. H_2O (10 ml) was added and the mixture was extracted with EtOAc (20 ml). pH of the aqueous phase was adjusted to 7 and the mixture was extracted with EtOAc (50 ml). The organic phase was dried over $MgSO_4$, filtered and evaporated. Purification by column chromatography (SiO2, EtOAc:Heptane 1:1). Yield: 66.2 mg (43 %), ^1H NMR (CDCl3, 300 MHz) δ 7.30 (m, 3H), 7.52 (m, 1H), 7.62 (m, 1H), 7.80 (m, 2H), 8.74 (m, 1H).

EXAMPLE 38



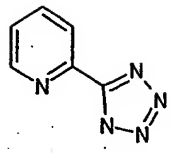
2-(2-Pyridyl)pyrazine. 2-Chloropyrazine (100 mg, 0.87 mmol) was dissolved in m-xylen (2 ml), 2-tri-*n*-butylstannylpyridin (354 mg, 0.96 mmol) was added followed by bis-(triphenylphosphine)palladium chloride (1.2 mg, 0.0017 mmol). The mixture was heated to 130 °C over night under nitrogen. The mixture was allowed to cool to room temperature. The crude mixture was purified by column chromatography (SiO2; EtOAc:Heptane 1:1). The product was dissolved in EtOAc (25 ml) and washed with aqueous HCl (pH ~ 3) (2 x 30 ml). The aqueous phase was adjusted to pH 8 with $NaHCO_3$ and extracted with EtOAc (2 x 20 ml). The organic phase was dried over Na_2SO_4, filtered and evaporated. Yield: 53 mg (38 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.39 (m, 1H), 7.87 (dt, J = 7.91, 1.69 Hz, 1H),8.38 (m, 1H),8.62 (m, 2H),8.74 (m, 1H), 9.66 (s, 1H).

EXAMPLE 39



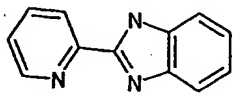
N-Hydroxy-pyridine-2-carboxamidine. Sodium (0.53 g 23 mmol) was dissolved in MeOH (15 ml), hydroxylamine hydrochloride (1.53 g 22 mmol) dissolved in MeOH (15 ml) was added, and stirred in ice bath for 1 hour. After filtration the solution was added 2-cyanopyridin (1.93 ml, 20mmol),and stirred at R.T. over night. The reaction mixture was reduced *in vacuo*. After cooling on ice the product precipitate. Filtered and washed with diethyl ether. Yield: 2.1 g (73 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.75 (br s, 2H), 7.34 (ddd J = 1.32 Hz, J = 4.9 Hz, J = 7.53 Hz, 1H), 7.73 (dt, J = 1.88 Hz, J = 7.54 Hz, 1H), 8.94 (dt, J = 1.13 Hz, J = 7.92Hz, 1H), 8.18 (br s, 1H), 8.58 (ddd, J = 0.95 Hz, J = 1.88 Hz, J = 4.9 Hz, 1H).

EXAMPLE 40



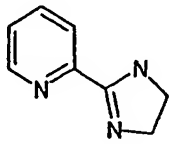
2-(5-Tetrazolyl)pyridyine. This chelator was prepared from the corresponding cyano-compound according to Example 15.

EXAMPLE 41



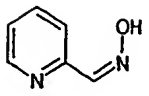
2-Pyridin-2-yl-1H-benzoimidazole. Picolinic acid (2.5 g, 20.3 mmol) was added THF (25 ml) and heated to reflux. Carbonyl diimidazole (3.6 g, 22.3 mmol) was added in portions, and the reaction was heated for 3 hours. After cooling to R.T., 1,2-phenylenediamine (2.2 g, 20.3 mmol) was added, and the reaction was stirred for 1 hour at R.T. Evaporated and dissolved in EtOAc, washed with water, dried and evaporated. The formed crystals were washed with diethyl ether and dried. Yield: 0.8 g.

EXAMPLE 42



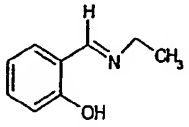
2-(4,5-Dihydro-1H-imidazol-2-yl)-pyridine. Picolinic acid (2.5 g, 20.3 mmol) was added THF (25 ml) and heated to reflux. Carbonyl diimidazole (3.6 g, 22.3 mmol) was added in portions, and the reaction was heated for 3 hours. After cooling to R.T., 1,2-ethylenediamine (1.4 ml, 20.3 mmol) was added, and a colorless precipitation was formed. The reaction was stirred for 1 hour at R.T. The solid was filtered off, washed with THF and dried. Yield: 1.3 g.

EXAMPLE 43



Pyridine-2-carbaldehyde oxime. 2-Pyridylcarbaldehyde (0.5 g, 4.7 mmol) and hydroxylamine hydrochloride (0.65 g, 9.4 mmol) was dissolved in ethanol (30 ml) followed by pyridine (0.76 ml, 9.43 mmol). The reaction was heated to reflux for 2 hours and 20 minutes. After cooling, the reaction was evaporated, the crude redissolved in EtOAc, washed with water, dried and evaporated, to give a colourless crystalline solid. Yield: 0.46 g (80 %).

EXAMPLE 44



2-Ethyliminomethyl-phenol. Salicylaldehyde (4.4 ml, 40.9 mmol) was dissolved in dry toluene (100 ml), and ethylamine (g) was bubbled through (3 x 5 minutes). Left at R.T. over night. Still starting material. The reaction was heated to 65 °C over night. Evaporated and distilled.

The following examples presented encompass naturally occurring as well as specifically engineered metal-ion binding sites in a number of different proteins representing several different classes of membrane proteins: 7TM proteins (examples being various G-protein coupled receptors), and 12TM proteins (example - the dopamine transporter) as well as an example comprising a soluble protein, Factor VIIa, the active form of the FVII protease.

The examples are chosen with the intent of illustrating the sequential and rational process through which small organic compounds, the metal-ion chelators, may be identified as ligands and subsequently optimized with respect to the affinity by which they recognize the protein targets.

Overall, the examples serve to illustrate how the activity of potential drug targets may be affected through interaction with small metal-ion chelators and importantly how the

present technology provides the opportunity to aim the active drug candidates towards functionally significant domains of the target. Throughout this section, 'the affinity' of the metal-ion chelator complexes refers to the ability of the complex to displace the binding of a radioligand and the potency of the metal-ion chelator complexes refers to the ability of the substances to activate or inactivate the drug targets.

I. Identification and binding Of Metal-Ions and Metal-Ion Complexes To Various Drug Targets With Natural Metal-Ion Sites

The examples compiled in this section illustrate how metal-ion binding sites may be identified in the native forms of various drug targets, and how these sites may be addressed by metal-ions in complex with certain chelators, as observed either through an effect on the binding affinity of a radioactive ligand or through a direct effect on activation or inactivation of the target.

Example I.1 – Identification of a naturally occurring metal-ion chelator binding-site in the 7TM leukotriene LTB4 receptor

The present example illustrates how the presence of a previously unnoticed, naturally occurring metal-ion binding site within a transmembrane segment of a 7TM receptor may be predicted through analysis of the nucleotide sequence of the gene coding for the protein and how it can subsequently be experimentally identified. Briefly, molecular models of 7TM receptors can be built based on the deduced amino acid sequence and identification of the seven transmembrane segments (eg.Unger at al. (1997) Nature 389: 203-206). In these molecular models, illustrated in the helical wheel diagram shown in Fig. 5, potential metal-ion sites can be identified by the presence of metal-ion binding residues, for example histidine, cysteine, or aspartate residues located in suitable relative positions, for example in an *i* and *i + 4* arrangement (i.e. with three residues in between) on a helical face within the so-called main ligand-binding crevice of the receptor between TM-II, III, IV, V, VI, and VII (Schwartz et al, (1996) Trends Pharmacol. Sci. 17: 213-216).

Methods – The leukotriene LTB4 receptor cDNA was cloned by PCR from a leukocyte cDNA library, built into an eukaryotic expression vector and introduced into COS-7 cells by a standard calcium phosphate transfection method. One day after transfection the cells were transferred and seeded in multi-well plates for assay. The number of cells plated per well was chosen so as to obtain 5 to 10% binding of the radioligand added. Two days after transfection the cells were assayed for the presence of [^{3}H]-LTB4 binding activity.

Radioligand was bound in a buffer composed of 50 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 0.1 % BSA, 100 mg/ml Bacitracin and displaced in a dose dependent manner by unlabelled LTB4 ligand. The assay was performed in duplicate for 3 hours at 4 °C, and stopped by washing twice in buffer. Cell associated, receptor bound radioligand was determined by the addition of lysis buffer (48% urea, 2% NP-40 in 3M acetic acid). The concentration of radioligand in the assay corresponds to a final concentration of 45 pM. The metal-ion chelating complex, 2,2'-bipyridine was added in a two-fold molar excess in order to ensure that no free metal-ion was present.

Results and discussion - As shown in the helical wheel diagram of the leukotriene LTB4 receptor (Fig. 5C), two Cys residues are located on the face of TM-III pointing inwards, i.e. towards the main ligand-binding pocket of the receptor (positions III:04, Cys^{93} and III:08, Cys^{97}). Theoretically these residues could constitute a metal-ion binding site. The actual presence of a naturally occurring metal-ion binding site in the leukotriene LTB4 receptor is demonstrated by the fact, that binding of the radioligand, [^{3}H]-LTB4 to the receptor expressed in COS-7 cells could be displaced by Cu(II), IC_{50} = 70 µM (Fig.5A). In agreement with the fact that the proposed metal-ion site is located in the main ligand-binding pocket of the receptor, i.e. with amble space towards the center of the receptor, the complex between the metal-ion and the chelator, 2,2'-bipyridine bound equally well as the free metal-ion, i.e. the 2,2'-bipyridine did neither impair nor improve the binding affinity (Fig.5A). As shown in Fig. 5B, Ala-substitution of Cys^{93} severely impaired the effect of the metal-ion chelator complex on LTB4 binding. Ala-substitution of Cys^{97} also clearly impaired the effect of the metal-ion complex. The combined substitution of both Cys residues totally eliminated the metal-ion chelator effect (Fig. 5B) demonstrating that these two residues on the central face of TM-III are involved in the binding of the metal-ion chelator complex. Thus, the two residues represent a naturally occurring intra-helical 'bis-Cys-site', which can be addressed with for example Cu(II) in complex with bipyridine..

Example I.2 – Identification of naturally occurring metal-ion binding site in the 7TM galanin receptor-1

The galanin receptor-1 (Gal-R1) provides yet another example of a 7TM receptor that contains naturally, endogenous metal-ion binding sites. Interestingly, this receptor has the potential of shifting its "chelation-mode" between two distinct chelation configurations.

Methods - The galanin receptor-1 cDNA was introduced into COS-7 cells by the standard calcium phosphate transfection method. The cells were transferred and seeded in multi-

well plates for assay one day following the transfection and the number of cells plated per well was adjusted for each individual (wild type and mutant) construct aiming at the binding of 5 to 10% of the radioligand present in the assay. Two days post-transfection the cells were assayed for the presence of [^{125}I]-Galanin binding activity. Radioligand was bound in buffer composed of 25 mM Hepes (pH 7.4), 2.5 mM $MgCl_2$, 100 mg/ml Bacitracin and displaced in a dose dependent manner by unlabelled ligand. The assay was performed in duplicate for 3 hours at 4 °C, and terminated by washing twice in ice-cold buffer, followed by the addition of lysis buffer (48% urea, 2% NP-40 in 3M acetic acid). The concentration of radioligand in the assay corresponds to a final concentration of 20 pM. Mutations were created in the cDNA by the PCR-directed overlap-extension method (Ho et al. (1989) Gene 77: 51-59).

Results and discussion - Binding of [^{125}I]-galanin to the Gal-R1 expressed in COS-7 cells is displaced by Zn(II) with an IC_{50} of 40 micromolar. From analysis of a molecular model of the Gal-R1 putative chelating residues in the receptor was identified and subjected to mutational analysis, Fig 6G. Importantly, by mutating either His267, His263 or His264 to Gln, Ala, Ala respectively, the apparent affinity for Zn(II) was significantly decreased suggesting that these residue are crucial for the observed high affinity of the wildtype Gal-R1 for Zn(II), Fig 62A, D, E. Interestingly, the double mutant [His263Q;His264Q] was virtually unresponsive to Zn(II), Fig 6.F. In contrast, mutating another residue in proximity of His263, His264 and His 267, in the molecular model, His112 to Ala, had no effect on the apparent affinity of Zn(II) suggesting that this residue does not participate in the chelation of Zn(II), Fig. 6.C. From analysis of a large number of naturally occurring metal-ion sites in proteins, it is well know for those skilled in the art, that an acidic residue if often located in proximity of the chelating histidines, where it by hydrogen bonding to one of the chelating histidines fine-tunes the histidine for chelation of the metal-ion. Consistent with this, mutating Glu271 to Gln, located one helical turn above the important His267, slightly decreased the apparent affinity for Zn(II), Fig. 6B.
A model consistent with these data, is depicted in Fig 6H, I. In binding mode I, the metal-ion is chelated primarily by residues His263 and His267, optimally spaced in an i, i+4 configuration in the helix. Upon mutation of His263, a new binding mode is observed, where the metal-ion is chelated by residues His264 and His267. The opposite is observed upon mutation of His264, when the binding mode shift solely to binding mode I. Alternatively, in the wildtype receptor, all three histidines 263, 264 and 267 chelates the metal-ion. However, in that model, the configuration of the histidines is not optimal for chelation and since the observed Zn(II) affinity is more consistent with a bidentate site, we favor a bidentate model.

These experiments demonstrate that naturally occurring intra-helical metal-ion sites in 7TM receptors can be identified, dissected by molecular analysis and addressed by a metal-ions or metal-ions in complex with chelators.

Example I.3 – Identification of naturally occurring metal-ion chelator binding site in the 12TM dopamine transporter.

In the literature, a naturally occurring allosteric metal-ion binding site has been demonstrated in the dopamine transporter, a membrane protein having supposedly 12 transmembrane spanning segments, 12TM (Norregaard et al EMBO J. 17: 4266-4273 (1998); Loland et al., JBC, 274, 36928-34, (1999)). Here Zn(II) binds in a two-component fashion to a tridentate metal-ion site composed of residues His^{193}, His^{375}, and Glu^{396} and thereby blocks dopamine transport. This effect of Zn(II) can be eliminated by mutational exchange of any of the three residues with a non-chelating residue.

Methods - The dopamine transporter cDNA was introduced into COS-7 by the standard calcium phosphate transfection method. Two days post-transfection the cells were assayed for [^{3}H]-Dopamine uptake activity. The uptake assays was performed in 25 mM Hepes pH 7.4, 120 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM ascorbic acid and 5 mM D-glucose and in the presence of various concentrations of unlabelled dopamine as indicated in the figures. The assay was performed in triplicate at 37°C for 10 minutes, and terminated by washing with buffer twice and the addition of lysis buffer (48% urea, 2% NP-40 in 3M acetic acid).

Results and discussion - As shown in Fig. 7A, 2,2'-bipyridine in complex with Zn(II) inhibits the transport of [^{3}H]dopamine by the dopamine transporter, transiently expressed in COS-7 cells, in a two component fashion, i.e. with IC_{50} values of 0.16 and 20 μM, corresponding to a slightly higher potency than the free metal-ion, which similarly acts in a two component fashion, i.e. with IC_{50} values of 2.2 and 338 ?M,. Importantly, the chelator bipyridine had no effect on the dopamine transport without being on complex with the metal-ion (Fig.7A). That the metal-ion chelator complex acts through the same site as the free metal-ion was demonstrated by the mutational exchange of residue His^{193} (Fig. 7B). Dopamine transport could be inhibited also by a structurally distinct class of metal-ion chelators, exemplified by 2-pyridylamidoxime,O-acetyl (compound 210), which like 2,2'-bipyridine does not affect dopamine transport by itself, but blocks dopamine transport with a potency approx. 10-fold higher than free Zn(II) and interestingly acts in a mono-

component fashion (Fig. 7C). This effect of the metal-ion chelator complex was eliminated by mutational substitution of His^{193} known to be involved in metal-ion binding (Fig. 7C). This substitution is known not to affect the transport of catecholamine (Norregaard et al (1998) EMBO J. 17: 4266-4273) indicating that the effect of the metal-ion chelator complexes is mediated through the binding to a site (i.e. the endogenous metal-ion site), which is different from the catecholamine binding site. Thus, the metal-ion chelator complexes act as blockers of transport through a novel allosteric molecular mechanism and could therefore serve as lead compounds in the development of a new type of transport blockers. It should be noted that the affinity of, for example 2-pyridylamidoxime,O-acetyl (compound 210) corresponds to even a very good lead compound found by simple screening.

The experiments presented in this section demonstrate that metal-ion chelator complexes of very different chemical structures can act as allosteric blockers of function - in these cases of either 7TM receptors or 12TM transporter proteins - through binding to naturally occurring metal-ion sites. Furthermore, it is shown that these compounds can bind with affinities similar to that of lead compounds found by conventional drug screening techniques. Thus, these metal-ion chelators can function as lead compounds in a chemical optimization process to obtain high affinity compounds acting as drug candidates.

II. Binding Of Metal-Ion Complexes In Engineered Metal-Ion Sites In Various Potential Drug Targets

Natural metal-ion sites are only found in a subset of potential drug targets. However, through mutagenesis it is possible to introduce metal-ion binding sites in proteins by introduction of metal-ion binding residues such as His, Cys, or Asp. The examples in the present section demonstrate how metal-ion complexes can bind to and affect the function of proteins after mutational engineering of metal-ion sites into the proteins.

Example II.1 – Binding of various metal-ion complexes to a library of inter-helical metal-ion sites engineered into the tachykinin NK1 receptor.

This example illustrates that different epitopes of a target protein - here a NK1 receptor - can be addressed by metal-ion chelator complexes, i.e. potential lead compounds for antagonists, after systematic mutational engineering of metal-ion sites into these different epitopes. Previously, a series of metal-ion sites have been built into the tachykinin NK1

receptor to probe helix-helix interactions, i.e. providing distance constraints in molecular models of the receptor (Elling et al. (1995) Nature 374: 74-77, Elling et al. (1996) EMBO J. 15: 6213-6219; Holst et al. (2000) Mol.Pharmacol. 58: 263-270). Here, such metal-ion sites are used as anchor points for potential lead compounds - i.e. metal-ion chelator complexes - for the development of receptor antagonists with different molecular mechanisms of actions.

Methods – The tachykinin NK1 receptor cDNA was expressed in COS-7 cells. Two days after transfection whole cells were assayed with respect to binding of radioactively labeled substance P ([^{125}I]-Bolton Hunter labeled Substance P), in displacement with substance P, $ZnCl_2$, $CuCl_2$ or various chelator complexes thereof present in a three fold molar ratio with respect to the metal-ion concentration. The zinc(cyclam) complex was prepared by co-incubation at 60 °C for one hour followed by overnight incubation at 37 °C. The assay was typically performed in 12 or 24 well plates. On the day of assay, the cells were washed with binding buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM $MnCl_2$, 0.1 % BSA, 0.1 % and Bacitracin (100 mg/ml). Unlabelled competitor ligand and radioligand (20,000 cpm – approximately 20 pM) was added to the cells in binding buffer and incubation continued for 3 hours at 4 °C. The assay was terminated by washing of the cells and lysis. The assay was performed in duplicate.

Results and discussion - Four different inter-helical metal-ion sites located between respectively TM-II and -III, TM-III and -V, TM-III and -VII, and TM-V and VI (Fig. 8, Table in Panel A) were here probed with metal-ion chelator complexes in competition binding experiments against [^{125}I]-substance P in COS-7 cells transiently transfected with the NK1 receptor. An increase in affinity from approx. 10-fold to around 50-fold was observed in the metal-ion site engineered receptors as opposed to the wild-type NK1 receptor for free Zn(II) as well as for Zn(II) in complex with either 1,10-phenanthroline or in complex with 2,2'-bipyridine (Fig.8A). Thus, single to double digit micromolar affinities were obtained for the metal-ion chelator complexes in these metal-ion site engineered receptors, corresponding to affinities observed for lead compounds in general found by conventional chemical screening. In the sites between TM-II and -III and between TM-III and -VII a similar increase in affinity was found for Cu(II) and Cu(II) in complex with the chelators as observed with the zinc-ions. However in the sites between TM-III and -V and between TM-V and -VI no increase or just a marginal increase in affinity was observed for copper and the copper-chelator complexes. Thus, different metal-ions can be exploited in different sites. In Fig.8B is demonstrated that an inter-helical bis-His site, in this case constructed between TM-V and TM-VI, can also be addressed by a metal-ion chelator complex where

the ion, in this case Zn(II), is bound in a circular chelator, here cyclam. Cyclam binds Zn(II) with a very high affinity, 3.2 x 10^{-16} M, which can be noted by the fact that the Zn(II)-cyclam complex has no effect on the wild-type NK1 receptor even at 10^{-3} M conc. i.e. an even smaller effect than the free metal-ion. Thus, the effect of the metal-ion chelator complex on the metal-ion site engineered receptor cannot be caused by the presence of free metal-ions.

The present example demonstrates that metal-ion chelator complexes can bind with suitable affinity, i.e. corresponding to ordinary lead compounds, in different parts of the main ligand-binding crevice of a 7TM receptor. This can be utilized, for example to target the lead compound and thereby subsequently the chemically optimized compound, i.e. the drug candidate, to bind and interact with different parts of the target molecule. In the present case, the metal-ion site between TM-II and -III can be used as anchor point for lead compounds addressing chemical interactions with wild-type residues located in the pocket between TM-I, -II, -III, and VII; whereas the metal-ion sites located between TM-III and -V and TM-V and -VI can be used as anchor points for chelating lead-compounds addressing residues in the pocket between TM-III, -IV, -V, -VI and -VII (see helical wheel diagram in Fig. 8C). The metal-ion site located between TM-III and -VII may in principle be used to address either of these pockets. This approach can be used to deliberately direct the chemical optimization process, i.e. the molecular recognition towards specifically interesting parts of the target protein in order to obtain for example selectivity for a certain receptor subtype or a certain member of a family of related proteins. For example, families of monoamine and adenosine 7TM receptors are generally very highly - if not totally - conserved in the binding pocket for the natural ligand, i.e. the pocket between TM-III, -IV, -V, -VI, and -VII; however, they differ more in the pocket between TM-I, -II, -III, and VII. Conventional drug discovery methods are for various reasons highly biased towards the binding pocket for the natural ligand. The present approach allows for deliberate targeting of the lead compound and thereby also the final drug candidate for allosteric sites, i.e. pockets or epitopes distinct from the one used by the natural ligand.

Example II.2 – Re-engineering of a metal-ion chelator binding site in the 12TM dopamine transporter.

In example I.3 , it was shown that the Zn(II)-bipyridine inhibited dopamine transport in a two-component fashion. This complicated type of interaction could hamper a subsequent further medicinal chemistry optimization of the chelator for high affinity interaction. In this

example, the naturally occurring metal-ion site was re-engineered by elimination of one part of the metal-ion binding site and by introduction a new metal-ion binding residue.

Methods - as in example I.3.

Results and discussion - Re-engineering of the metal-ion site in the dopamine transporter was done by eliminating His^{193}, i.e. the residue found in the proposed extra-cellular loop 1, by substitution with a Lys residue and by introduction of an alternative metal-ion chelating His residue either in exchange for Glu^{396} located at the extra-cellular end of TM-8 or in exchange for Val^{377} located in TM-7 (Norregaard et al. Biochemistry, 26, 15836-46, (2000). Both of these introduced His residues are located in a potentially favorable configuration for participating in metal-ion binding with His^{375} in TM 7. As shown in Fig. 9, in both cases - [H193K;E396H] and [H193K;V377H] - more mono-component interaction curves were obtained for the metal-ion chelator complex in the re-engineered transporter mutants as compared to the wild-type transporter protein. This example demonstrates that a natural metal-ion site can successfully be re-engineered to create a less complex molecular or pharmacological phenotype. In a subsequent medicinal chemical optimization process such re-engineered metal-ion sites will be used in parallel with the natural site during the screening of chemical libraries.

In biological target molecules in general, more than one version of an engineered metal-ion site can in a similar fashion be used in parallel in the screening process in order to exploit the chemical libraries more efficiently. This approach enables each compound to contact, for example the same amino acid side chain located on an opposing transmembrane helix in more than one configuration.

Example II.3 – Probing different metal-ions in an engineered Bis-His TM-V Kappa opioid receptor and evaluating the chelator strength of various metal-ion chelators.

This example illustrates the importance of testing various metal-ions in a metal-ion binding site in for example a 7TM receptor, probing the selectivity of the site. This was done in the Kappa opioid receptor in which a metal-ion binding site was engineered by substituting the endogenous residues for histidine at positions V:01 and V:05 in TM-V (i.e. [D223H;K227H]).

Methods - Mutations were created in the rat Kappa opioid receptor cDNA by the PCR-directed overlap-extension method (Ho et al. (1989) Gene 77: 51-59). The Kappa opioid

receptor cDNA was expressed in COS-7 cells. Two days after transfection whole cells were assayed with respect to binding of radioactively labeled [^{3}H]-Diprenorphine in displacement with Diprenophine, or various metal-ions. The assay was typically performed in 12 or 24 well plates. On the day of assay, the cells were washed with binding buffer (50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.1 % BSA, 0.1 % and Bacitracin (100 mg/ml). Unlabelled competitor ligand and radioligand (ca. 50 pM) was added to the cells in binding buffer and incubation continued for 3 hours at 4 °C. The assay was terminated by washing of the cells and lysis. The assay was performed in duplicate.

For evaluation of the relative chelator strength for Zn(II), a fluorescence based assay was performed. Briefly, the chelator of choice was dissolved in 20 mM sodiumphosphate buffer pH 7.4, 100 mM NaCl, 200 micromolar mercaptoethanol, 20 nM $ZnCl_2$ and 1 micromolar FluoZin-3 (Molecular Probes) and incubated for two hours at room temeperature after which the fluorescence of fluozin-3 was measured in a NovoStar reader (BMG).

Results and discussion – As shown in Fig. 19, the engineered metal-ion site, shows a clear selectivity for certain metal-ions over others. For example, whereas Zn(II) has an IC50 of 1.1 micromolar and Cu(II) an IC50 value of 4 micromolar, Gd(II) is a significantly lower apparent affinity of 42 micromolar.

The selectivity of a metal-ion site in a biological target may be used in combination with the selectivity displayed by various metal-ion chelators, Fig. 10, to fine tune the binding of the complex to the metal.ion site in the biological target at for example the desired affinity range.

The experiments presented in this section demonstrate that metal-ion chelator complexes can act as blockers of the function of biological target molecules - in these cases of either 7TM receptors or 12TM transporter proteins - through binding to metal-ion sites introduced by mutagenesis. Furthermore, these compounds can bind with similar affinity as lead compounds found by conventional drug screening techniques. Thus, these metal-ion chelators can function as lead compounds in a chemical optimization process to obtain high affinity compounds acting as drug candidates.

III. Increasing The Affinity / Potency / Efficacy Of The Metal-Ion Chelator Complexes Through Chemical Modifications Of The Chelator Molecule

In the present collection of examples, the metal-ion chelators are considered as being bi-functional compounds, i.e., being composed of a metal-ion chelating moiety and a variable chemical moiety which interacts positively or negatively – depending on the chemical

recognition - with spatially surrounding parts of the biological target molecule to which the chelator binds through either a natural or an engineered metal-ion site.

Example III.1 – Structure-activity relationship of antagonist metal-ion complexes in the leukotriene LTB4 7TM receptor.

As discussed in examples I.1 the human leukotriene LTB4 receptor has a metal-ion site located between Cys^{93} and Cys^{97}, located in TM-III.

Methods – as in examples I.1.

Results and discussion - A small library of commercially available 1,10-phenanthroline analogs in complex with Cu(II) were tested in competition binding against [^{3}H]-LTB4 on the LTB4 receptor expressed in COS-7 cells. Interestingly, a significant increase in affinity was observed for compound TM-270, whereas a significant decrease in apparent affinity was observed for compound TM-401, demonstrating SAR on the LTB-4 receptor (Fig. 11).

Example III.2 – Structure-activity relationship of antagonistic metal-ion complexes in the metal-ion site engineered tachykinin NK1 7TM receptor

The tachykinin NK1 receptor, which currently in the industry is a major putative target for the development of anxiolytic, antidepressive, as well as anti-emetic drugs, is here used as an example of a biological target molecule, in which an engineered metal-ion site can be used as an anchor point for the discovery and development of antagonistic drug candidates. As demonstrated in example II.1 a number of metal-ion sites could be built into the NK1 receptor and addressed by metal-ion chelator complexes competing for binding against radioactive substance P through interactions at different sites in the main ligand-binding pocket of the receptor, depending on the location of the metal-ion. Here, structure-activity relationships are demonstrated for a series of chelator analogs in two of these sites, i.e. the site between V:05 and VI:24 and the site between III:08 and VII:06.

Methods – as in example II.1.

Results and discussion – Many of the chemical variations of the variable part of the chelator were tolerated in the structure of the NK1 receptor when bound to the engineered metal-ion sites in complex with Zn(II). However, as demonstrated in Fig 12, clear differences were observed for some of the analogs in the two selected sites. Thus, 2,9-

bis(trichloromethyl)-1,10-phenanthroline (compound 135) and 1,10-phenanthroline-5,6-dione (compound 175) bound 6- and 10-fold better than 1,10-phenanthroline in the [HisV:05,HisVI:24] site, but almost similar to 1,10'-phenanthroline in the [HisIII:08;CysVII:06] site - all in complex with Zn(II). In contrast the 5-phenyl-1,10-phenanthroline (compound 134) was 7-fold more potent in the [HisIII:08;CysVII:06] site than phenanthroline but only slightly more potent in the [HisV:05,HisVI:24] site - again all in complex with Zn(II). It should be noted here, that 5-phenyl-1,10-phenanthroline (compound 134) bound like 1,10'-phenanthroline in the galanin receptor, but was totally inactive in the leukotrien LTB4 receptor (see Fig. 11).

This example together with the previous example demonstrate, that relatively minor chemical modification of the variable, "non-metal binding" part of the chelator molecule can alter the recognition and antagonistic property of the metal-ion chelator complex both in biological target molecules having naturally occurring metal-ion sites as well as in molecules into which metal-ion sites have deliberately been engineered. Importantly, increases in affinities are observed demonstrating that the metal-ion chelators can be utilized as lead compounds in a drug discovery process towards high affinity compounds.

Example III.3 – Structure-activity relationship and dependency of specific metal-ion chelating residues of *agonist* metal-ion complexes in the metal-ion site engineered beta$_2$-adrenergic 7TM receptor.

It is generally known in the field that while it is possible to find *antagonistic* lead compounds and optimize these for high affinity through medicinal chemistry efforts in many biological target molecules, it is generally much more difficult to find and develop *agonist* compounds, that is compounds, drug candidates, which activate the biological target molecule. The present example demonstrates how an engineered agonistic metal-ion site can be used as anchor-point for the development of *agonists* in a 7TM receptor. Furthermore the present example demonstrates the importance of testing various combinations of chelating aminoacids in the same positions when engineering a metal-ion bindings site, as the specific positions, of which the site is composed, may only define a metal-ion binding site for some combinations (e.g. His-Cys or Asp-Cys) and not others (e.g. His-His, Glu-His etc.).

Methods – Mutations were created in the beta2-AR cDNA by the PCR-directed overlap-extension method (Ho et al. (1989) Gene 77: 51-59). The beta2-AR cDNA was expressed by transient transfection into COS-7 cells. Two days after transfection the cells were

assayed for intracellular levels of basal and ligand-induced cyclic AMP. The assay employed is essentially as described in Solomon et al (Anal.Biochem. (1974) 58: 541). Labelled adenine ([^{3}H]adenine, Amersham TRK311) was added to cells seeded in 6-well culture dishes. The following day the cells were washed twice with HBS buffer [25 mM Hepes, 0.75 mM NaH_2PO_4, 140 mM NaCl (pH 7.2)] and incubated in buffer supplemented with 1 mM 3-isobutyl-1-methylxanthine (Sigma I-5879). Agonists were added and the cells were incubated for 30 min at 37 °C. The assay was terminated by placing the cells on ice and aspiration of the buffer followed by addition of ice-cold 5% trichloroacetic acid containing 0.1 mM unlabelled camp (Sigma A-9062) and ATP (Sigma A-9501). Cyclic AMP was then isolated by application of the supernatant to a 50W-X4 resin (BioRad) and subsequently an alumina resin (A-9003; Sigma) eluting the cyclic AMP with 0.1 M imidazole (Sigma I-0125). Determinations were done in duplicate. For the wash experiment the [F289C;N312C]β_2-AR construct was stimulated with 100 μM copper-(2,2'-bipyridine)3 complex at time zero; after 10 minutes cells were washed two times whereafter half of the cells were either re-challenged with 100 μM copper-(2,2'-bipyridine)3 or with buffer. The basal cAMP accumulation was monitored in parallel. The level of cAMP production was sampled for each 5 minute interval.

Results and discussion – The inventors have previously demonstrated that Cys-substitution of Asn^{312} (AsnVII:06) in TMVII in the beta2-adrenergic receptor creates a bi-dentate metal-ion binding site with AspIII:08 at which metal-ion chelator complexes such as 1,10-phenanthroline and 2,2'-bipyridine in complex with either Zn(II) or Cu(II) can bind and act as agonists for the receptor (Elling et al. PNAS 1999, 15: 6213-6219). As shown in Fig. 13 and Fig. 20 an extended version of this site including also a substituted residue, Phe^{289} (PheVI:16) located in the important TM-VI, or a site only bridging positions III:08 in TM-III and position VI:16 in TM-VI, metal-ion chelator complexes, in this case Cu(II)-1,10-phenanthroline and Cu(II)-2,2'-bipyridine display higher agonistic efficacy and in some cases potency, than in the TM-III to TM-VII site. The free metal-ion or the chelator by itself has no stimulatory effect in the metal-ion-site engineered receptor (Fig. 20). That the agonistic effect of the metal-ion chelator complex is not caused by some kind of covalent modification of the receptor - for example oxidation – is shown in Fig. 14A, where a simple washing experiment demonstrates how the stimulatory effect quickly disappears, when the metal-ion chelator is removed, while the stimulation continues if the metal-ion chelator complex is re-added. When a library of bipyridine analogs, substituted either in the 3, 4 or 5 position were tested for agonistic activity in the [F289C] beta-2 adrenergic receptor, many were found not to be active, or to be less active than 2,2'-bipyridine, Fig. 15C, while some were shown to be as potent as bipyridine itself (Fig . 14B and 15C). Importantly, a

compound such as 2,2'-di(4-(benzimidazol-2-yl)-quinoline),(compound 85) was found to stimulate signal transduction as determined in cAMP accumulation in the metal-ion site engineered receptor with a 21-fold improved potency, i.e. EC_{50} = 470 nM as compared to 2,2'-bipyridine. Furthermore, a number compounds such as for example copper-(TM-325)2 had a higher efficacy compared to copper-(2,2'-bipyridine)2, Fig.15C.

The importance of testing various combinations of chelating amino acids in the same positions, when engineering a metal-ion binding site is shown in Fig. 20 and Fig. 13A. Whereas a given combination of aminoacids in positions 113 (III:08) and 312 (VII:06), for example histine, cysteine respectively define an agonistic metal-ion binding site, other combinations, for example histidine, histidine respectively, does not. Similarly, whereas engineering of an agonistic metal-ion binding site composed of chelating residues at positions 113 (III:08) and 289 (VI:16) is successful with the combination aspartate (utilizing the naturally occurring aspartate 113 (III:08)) and cysteine respectively, the combination histidine, histidine respectively is unsuccessful. Furthermore, whereas more than one combination of amino acids may successfully define a metal-ion binding site at the same positions, the individual successful combinations may have different properties reflected in for example the specific pharmacology. In Fig. 20 this is demonstrated by the differing agonistic efficacy and cation selectivity of constructs involving postion 113 (III:08) and position 289 (VI:16).

This example demonstrates, that the variable, non-metal-ion binding part of the chelators can be modified to create nanomolar affinity agonists in metal-ion site engineered biological target molecules. Such a compound could serve as an intermediate "chemical stepping-stone" in the process of developing high affinity agonists for the metal-ion site engineered receptor. And, similarly agonistic metal-ion sites can be engineered into other 7TM receptors and other biological target molecules in general to serve as anchor points for the initial identification as well as the initial optimization process for agonist leads for such target molecules. Furthermore, this example demonstrates the importance of testing various combinations of chelating amino acids in the same positions when engineering a metal-ion binding site, as some combinations may successfully define a site and other combinations may not, and as the various successful combinations may have different properties, reflected in for example their specific pharmacology or cation selectivity.

Example III.4 – Structure-activity relationship of antagonistic metal-ion complexes in a soluble protein, the enzyme factorVIIa (FVIIa)

The previously presented examples have all represented membrane proteins, which obviously constitute a very large group of biological target molecules for medical drugs. In the present example, Factor VIIa, i.e. the active form of the FVII protease involved in the coagulation cascade is used to demonstrate that metal-ion chelator complexes can modulate the function of a soluble protein, in this case an enzyme which is known to possess an appropriate, allosteric metal-ion site (Dennis et al. Nature (2000) 404: 465-470).

Method – The amidolytic activity of Factor VIIa (FVIIa) was measured by the incubation of 2.5 µl FVIIa (100 nM final concentration, obtained from American Diagnostica), 2.5 µl ligand and 4 µl substrate (10 mM, S2288 obtained from Chromogenix) in 42.5 µl buffer (50 mM Hepes pH 7.4, 1 mM $CaCl_2$, 100 mM NaCl, 0,02% Tween 20). The assay was performed in 96-well plates (Costar). Incubation was performed at room temperature for five hours with absorbance read every 10 minutes.

Results and discussion – As shown in Fig. 16A, 2,2'-bipyridine without metal-ions has no effect on the activity of FVIIa; however in complex with Zn(II), 2,2'-bipyridine inhibits the enzymatic activity with a 100 micromolar affinity. Many bipyridine analogs act with a similar potency as the basic chelator, however for example Zn(II)-4,4'-di-terbutyl-2,2'-dipyridyl (compound 180) inhibits FVIIa enzyme activity with an 8.5-fold *increased* potency as compared to Zn(II)-bipyridine (Fig. 16A). In contrast Zn(II)- 4,4'-di-terbutyl-2,2'-dipyridyl (compound 180) inhibits LTB4 binding to the LTB4 receptor with a potency which is 10-fold *lower* than Zn(II)-bipyridine alone (Fig. 16B). As shown in Fig. 16C, 1,10-phenanthroline had no effect on FVIIa activity by itself, however in complex with Zn(II) 1,10-phenanthroline inhibits the enzyme activity with a potency of 110 micromolar. As with 2,2'-bipyridine, many phenanthroline analogs act with a potency similar to or lower than 1,10-phenanthroline itself (data not shown); however, for example 2,9-bis(trichloromethyl)-1,10-phenanthroline in complex with Zn(II) inhibits FVIIa activity with increased potency as compared to Zn(II)-1,10-phenanthroline (Fig. 16C).

Most enzyme inhibitors act by binding at - or near by - the active site of the target molecule. However, as recently demonstrated for FVIIa, very efficient inhibition can be obtained also by binding instead at exosites or allosteric sites located far away from the active site in the biological target molecule (Dennis et al. Nature (2000) 404: 465-470). The method described here can be utilized to specifically target the lead compound and thereby the final drug candidate to act at allosteric sites in the target molecule, as the binding site is determined by the site at which the anchoring metal-ion site is engineered.

Inhibition of enzymes and proteins in general at allosteric sites is particularly interesting since the active site often is relatively similar in enzymes belonging to a particular protein family, for example kinases or phosphatases, which means that it can be difficult to obtain selectivity of drugs acting at the active site. This is not the case with drugs acting at allosteric sites.

Example III.5 – Structure-based optimization of metal-ion chelators for secondary interactions in the CXCR4 receptor and other biological target molecules.

The previous examples in this session have demonstrated, that it is possible to obtain both decreased, but importantly, also *increased* affinity by modifying the variable, non-metal binding part of metal-ion chelators, which in various biological target molecules bind to either natural or engineered metal-ion sites. These examples were gathered mainly from screenings of commercially available, small libraries of chelator analogs. In the present example it is described how the process of increasing the affinity or potency of the metal-ion chelator can be performed in a deliberate structure based fashion in this case through the establishment of a charge-charge interaction. The metal-ion-mediated binding of the metal-ion chelator is here considered as being the "primary interaction point" or the anchor point, while the subsequent establishment of other chemical interactions is considered to be "secondary interaction points".

Methods –The cDNA coding for, for example the CXCR4 chemokine receptor can be expressed in COS-7 cells as described for other 7TM and 12TM proteins previously. Metal-ion sites may be engineered through PCR-directed mutagenesis and the functional activity of the receptor be tested for instance by (established) binding experiments employing the radiolabelled ligand, [^{125}I]-SDF1α.

Results and discussion – The inventors have demonstrated that Asp^{171} (AspIV:20) located at the extracellular end of TM-IV on the face pointing inwards, towards the main ligand binding crevice of the CXCR4 receptor is exposed and can be used as attachment site for the positively charged cyclam ring of non-peptide bicyclam antagonists for this receptor. Metal-ion binding sites will be introduced in the CXCR4 receptor in the spatial vicinity of AspIV:20 by introduction of a His residue at position V:01 which will form a bis-His metal-ion binding site with the naturally occurring HisIII:05 in the CXCR4 receptor - as previously demonstrated in the NK1 receptor (Elling et al. EMBO J. (1996) 15: 6213-6219). Similarly an intra-helical bis-His site will be introduced between residues V:01 and V:05 through introduction of two His residues at these positions and between III:05 and

IV:24 through His substitution at position IV:24. Thus three metal-ion sites will be constructed all within few Å's of AspIV:20 (see helical wheel diagram in Fig. 17). A small library of 1,10-phenanthroline, 2,2'-bipyrdine and 8-mercaptoquinoline analogs will be obtained or synthesized in which amino-methyl, amino-ethyl, amino-propyl, and aminobutyl will be placed in either the 2, 3, 4, or 5 positions and a similar small library where the same substituents will be placed in either the 3, 4, or 5 position of bipyridine will similarly be constructed. In a typical experiment, these libraries of amino-substituted chelators will be tested in complex with either Zn(II) or Cu(II) in the metal-ion-site engineered CXCR4 receptors, and the compounds ability to inhibit the binding of ^{125}I-SDF1a or the binding of [^{125}I]-12G5 monoclonal antibody or the ability of the compounds to inhibit the signal transduction mechanism induced by SDF-1a will be tested as performed for metal-ion chelators in the previous examples described above. Due to the spatial proximity as well as the relative conformational flexibility of the system, several of these compounds will in several of the sites have the opportunity of forming a salt-bridge between the amino function of the amino-substituted metal-ion chelator and the carboxylic acid function of Asp^{171} (AspIV:20). This formation of a secondary interaction will be quantified as an increased affinity or an increased potency of the metal-ion complex of the amino-substituted chelator in comparison to the corresponding metal-ion complex of the non-substituted phenanthroline or dipyridine. Due to the relatively high energy in the charge-charge-interaction a considerable increase in affinity or potency will be observed. The molecular interaction mode of the amino-substituted chelator(s) will be confirmed through mutational substitutions of Asp^{171} with Asn, Ala and other residues. Depending on the structure of the most optimal amino-substituted analog(s) a second and third round of analogs will be synthesized which conceiveably will present an appropriate basic moiety in a more conformationally constrained fashion.

These mini-libraries of amino-substituted metal-ion chelators can be utilized in several biological target molecules, which present Asp or Glu residues in an appropriate fashion. For example, in the CXCR4 receptor Asp^{262} (AspVI:23) is equally available as Asp^{171} for interaction as previously described (Gerlach et al.). Similarly AspIII:08 is conserved among monoamine receptors and, for example opioid and somatostatin receptors and this residue is a known interaction point for amine functions (Strader et al (1991) 266: 5-8). These and other acidic, potential secondary interaction points for amino-substituted metal-ion chelators can be addressed through construction of a small number of metal-ion sites placed in their spatial vicinity - as described above for Asp^{171} (AspIV:20). Similarly amino-functions in a biological target molecule – for example, epsilon amino groups of Lys residues –can be addressed by, for example mini-libraries of tetrazol substituted metal-ion

chelators. As described, charge-charge interactions will initially be pursued for establishing secondary interactions for the metal-ion chelator lead compounds. However, other types of weaker interactions such as hydrogen-bonds, amino-aromatic interactions, aromatic-aromatic interactions, aliphatic hydrophobic interactions, cation-pi interactions, van der Walls interactions etc. will also be exploited in a similar, systematic fashion as described above for the charge-charge interactions.

Example III.6 – Increasing the affinity of the metal-ion chelator in the [HisV:01;HisV:05] engineered LTB4 receptor

This example demonstrates how complexes with increased affinities may be identified on an engineered metal-ion binding site in the leukotriene B4 receptor.

Methods – see example I.1

Results and discussion – A library of approximately 500 2,2'-bipyridine analogues were synthesized and screened in combination with copper on the LTB4 receptor containing an engineered metal-ion binding site at the top of TM-V, [HisV:01;HisV:05]. From this screen, a compound was identified with a 50-fold increase in affinity compared to the unsubstituted scaffold, 2,2'-bipyridine, Fig. 18. Importantly, the compound had no increase in affinity on the wildtype LTB4 receptor, Fig 18. This illustrates how the affinity of a metal-ion complex may be increased by chemical modification of the chelator molecule, enabling secondary interactions to be identified.

In the present section, a 7TM receptor is for convenience used as an example of a biological target molecule. In this system, very useful molecular models are available, which have been refined and have allowed for, for example the construction of intra- and especially inter-helical metal-ion sites. However, due to lack of, for example an array of suitable X-ray structures of this or similar targets in complex with agonists and antagonists it is not possible to apply classical structure-based drug design methodology in full. Nevertheless, for example in these membrane proteins the present method does to a certain degree compensate for the lack of knowledge of the detailed 3D structure of the target molecule by anchoring the lead compound and thereby creating a fix-point for the subsequent medicinal chemical optimization point guided by the molecular models.

The approach described above could be further helped and guided by detailed knowledge of the 3D structure(s) of the biological target molecule, preferentially determined in

complex initially with the un-substituted metal-ion chelator and subsequently in complex with the chemically modified metal-ion chelator in which attempts have been made to establish first one secondary interaction and subsequently further secondary or tertiary interactions. For some biological target molecules such as soluble proteins this can be achieved through for example crystallization and standard X-ray analysis procedures or through, for example NMR analysis of the complex in solution again using standard procedures. Here, the method can take advantage of methods developed for structure-based drug discovery in general. This would make it possible to apply classical structure-based approaches such as structure-based library design for the establishment of secondary and tertiary interaction sites for the lead compound in the target molecule. However, it should be noted, that a major advantage and difference of the present method is, that the lead compound is anchored to a particular site and thereby to a certain degree in a particular conformation in the biological target molecule through binding to the bridging metal-ion site while the compound is being optimized for chemical recognition with the target molecule.

Also it should be noted that through the application of a more-or-less flexible spacer in between the metal-ion chelating moiety and the so-called variable chemical moiety of the test compound it becomes possible to probe for interaction or binding to structurally and functionally interesting epitopes of the biological target molecule with variable chemical moieties, which due to their intrinsic low affinity would not be detectable in the analytical systems on their own; but, which - due to the local high concentration of these created by the binding of the tethering metal-ion chelating moiety to the metal-ion site - now are detected.

Example III.7 - Improving the affinity of a stable test compound for a metal-ion site engineered receptor previously employed for creating a genetically modified test animal through establishment of second site interactions in the receptor by chemically modifications of the chelator.

In the previous example it was demonstrated that stable test compounds – i.e. metal-ion chelator complexes - acting as efficient inhibitors in metal-ion site engineered receptors can be made through the use of, for example Pd(II) as the metal-ion. In order for a test compound to efficiently and selectively block the function of a metal-ion site engineered biological target molecule in an in vivo setting it needs besides being stable also to have a sufficient high affinity for the metal-ion site engineered receptor. In the present example it is demonstrated that the affinity of such a stable metal-ion chelator complex can be

increased considerably through chemical modification of the side-chain of the chelator establishing second site interactions with the receptor.

Method – see example I

Results – As shown in Fig. 21 (upper panel to the left), bipyridine or rather 4,4-dimethyl-bipyridine in complex with Pd(II) does not affect the signaling of the wildtype RASSL receptor in response to the non-peptide agonist ICI199,441 and it has an inhibitory potency above 10^{-5} M in the RASSL receptor with an engineered metal-ion site between the natural AspIII:08 and the mutated CysVII:06 (the results for the Pd(II) complex with 4,4-dimethyl-bipyridine is shown in the figure to indicate that minor substitutions to the bipyridine chelator had no beneficial effect on the potency of the metal-ion chelator complex). When the bipyridine chelating moiety is decorated with a ethoxycarbonylcyclohexyl moiety connected via an amide bond (compound 433) then its Pd(II) complex is still inactive in the wildtype RASSL receptor, however now the potency of the Pd(II)complex of compound 433 has a potency of 200 nM (Fig. 21, lower left panel). Thus, the side chain which has been placed on the bipyridine cchelator has established second site interactions with the metal-ion site engineered RASSL receptor which has increased its affinity as here determined by the more than 100 fold increase in its potency in blocking the ICI199,441 stimulation of the receptor. This example demonstrates the general principle in increasing the affinity of a test compound through the establishment of second site interactions by chemical modifications of the metal-ion chelator. This can be achieved through non-covalent interactions as shown in the present example or through the establishment of covalent bonds, for example with a natural or an introduced, reactive Cys residue.

Example III.8 – Structure-based optimization of metal-ion chelators to use as antagonists in "pharmacological knock-out" experiments.

The approach described in the previous examples will be used as (a) step(s) in the drug development process in general to increase the affinity of lead compounds for the biological target molecule through establishment of chemical recognition between the ligand and structural elements found in the wild-type target molecule, i.e. in the unmodified vicinity of the engineered metal-ion site. However, the method will also be used for example to increase the affinity and specificity of metal-ion chelator compounds to be used in pharmacological knock-out applications. This procedure has in principle been described previously (Elling et al. (1999) Proc.Natl.Acad.Sci.USA 96: 12322-12327);

however only for basic metal-ion chelating agents. Briefly, the method is based on the introduction of a silent metal-ion site in a potential drug target, i.e. creation of a metal-ion site in which the mutations do not affect the binding and action of the endogenous ligand for the receptor. When such a metal-ion site engineered receptor is introduced into an animal by classical gene-replacement technology, i.e. exchange of the endogenous receptor with the metal-ion site engineered receptor, then the animals will develop normally without any development of compensatory mechanisms, which otherwise frequently impair the interpretations of the phenotypes in classical gene knock-out technology. In the adult animals or whenever it is found appropriate the animals are then treated with an appropriate metal-ion-chelating agent which then will act as an antagonist and turn off the function of the metal-ion site engineered receptor. Currently, this approach is impaired by the fact, that the generally available metal-ion chelating agents only will bind with at best micromolar affinity to the metal-ion site engineered biological target molecule, which will give similar micromolar or lower antagonistic potencies. These relatively low potencies and the relative low specificity of the basic test compounds impairs the general applicability of the technology due to simple pharmacokinetic and toxicology problems.

By applying the technology described in the previous example and in the previous examples in general, it will be possible to increase the affinity of metal-ion chelators significantly, which will make it considerably more easy to reach therapeutic, efficient antagonistic concentrations of the metal-ion chelator in the animals and also to increase the "therapeutic window" due to the higher degree of selectivity of the compounds caused by the establishment of more than one molecular interaction point. Establishment of just a single suitable charge-charge interaction will increase the affinity of the metal-ion chelator by 10 to 100-fold or more. This will be performed as an example in the so-called RASSL a modified kappa-opioid receptor, which previously has been used in gene-knock out experiments (Redfern et al. Nat. Biotechnol. (1999) 17:165-169). By introduction of metal-ion sites, for example between TM-V and TM-VI or between TM-VI and TM-VII or between TM-II and TM-III or between TM-III and TM-VII in a kappa-opiod RASSL molecule and through screening of, for example the mini-library of amino-substituted metal-ion chelators it will be possible to select a nano-molar affinity antagonist because of the formation of a secondary charge-charge interaction with AspIII:08, i.e. the Asp in TM-III corresponding to the amine-binding Asp in monoamine receptors.

IV. Optimization Of Compounds On The Wild-Type Biological Target Molecule

In the case, where the initial binding of the metal-ion chelator was obtained through mutational introduction of an anchoring metal-ion site in the biological target molecule, a final step of optimization will have to be performed to obtain high affinity binding or potency on the wild-type target molecule without the metal-ion bridge. Through the methods described in the previous experiments, the metal-ion chelator lead compound will gradually be optimized for interactions with chemical groups in the biological target molecule spatially surrounding the metal-ion site - i.e. interactions with chemical groups found also in the wild-type target molecule. Thus, the test compound will gradually increase its affinity not only for the metal-ion site engineered molecule but also for the wild-type biological target molecule. When two to three secondary interaction points have been established, the affinity of the test compound for the wild-type target molecule, which is being tested in parallel with the metal-ion site engineered molecule, will have reached micro-molar affinities, i.e. a lead compound on the wild-type target molecule has been created. At this point one or more of the following three approaches will be followed: 1) structure-based further chemical optimization of the compound in general aiming at improving recognition at various known chemical moieties of the target molecule; 2) structure-based further chemical optimization of the compound at which the "metal-ion site bridge" is exchanged by a more classical type of chemical interaction with the residue(s) which had been modified to create the metal-ion site in the biological target molecule. Here advantage can be taken of the fact that the geometry of the metal-ion site anchor is well known in general and, that relatively limited structure-based libraries can be established to create a new type of interaction; 3) further chemical optimization of the compound through more-or-less random generation of chemical diversity in general in the compound.

The above-given examples describe specific methods that can be employed to practice the present invention. Based on the details given a person skilled in the art will be able to devise alternative methods at arriving in the same information using the concept of the invention. However, the examples are not to be construed to limit the invention in any way.

Examples of compounds used in examples herein

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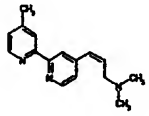
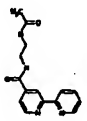
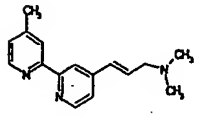
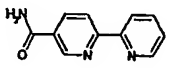
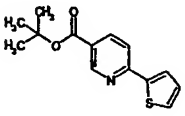
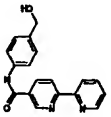
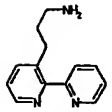
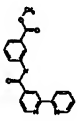
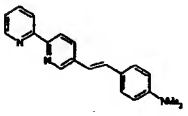
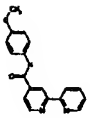
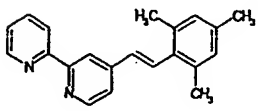
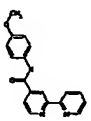
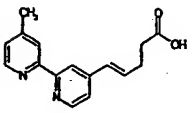
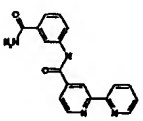
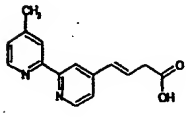
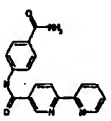
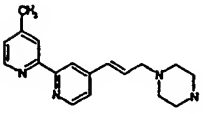
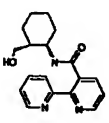
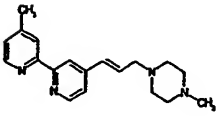
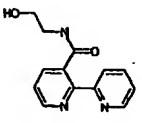
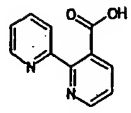
	TM482		TM279
	TM483		TM241
	TM486		TM263
	TM487		TM293
	TM489		TM276
	TM492		TM276
	TM490		TM304
	TM491		TM307
	TM505		TM333
	TM506		TM334

Examples of compounds used in examples herein



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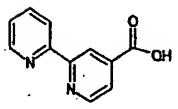

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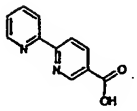

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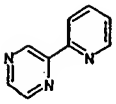

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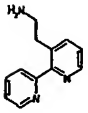

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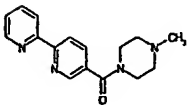

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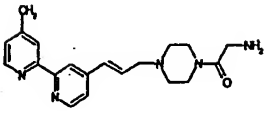

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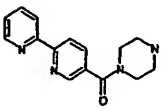

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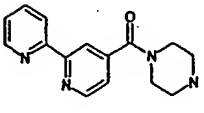

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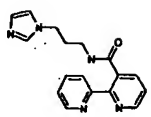

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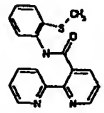

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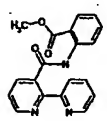

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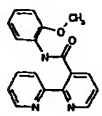

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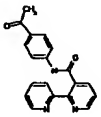

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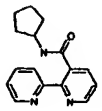

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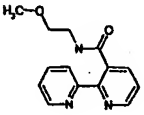

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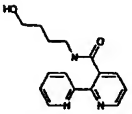

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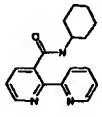

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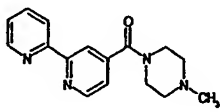
Examples of compounds used in examples herein



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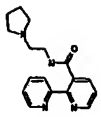

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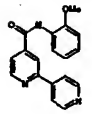

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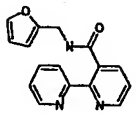

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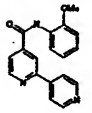

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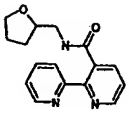

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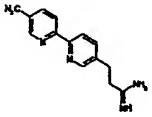
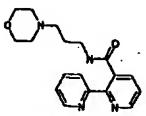
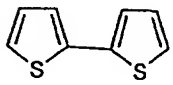


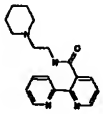
TM521



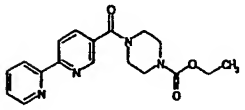
TM347



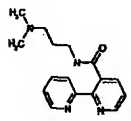
TM316



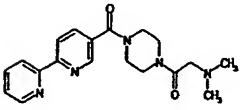
TM348



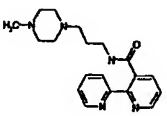
TM528



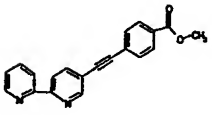
TM349



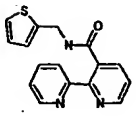
TM529



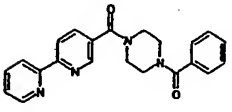
TM350



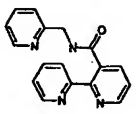
TM620



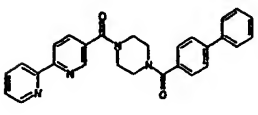
TM351



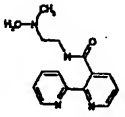
TM538



TM352



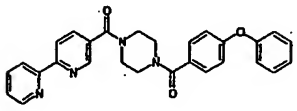
TM542



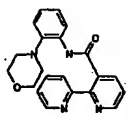
TM353

Examples of compounds used in examples herein

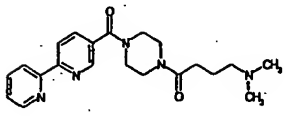
TM539



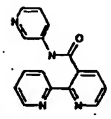
TM354



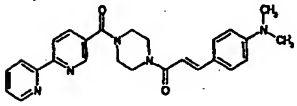
TM546



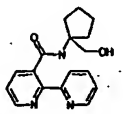
TM355



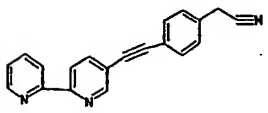
TM547



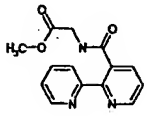
TM356



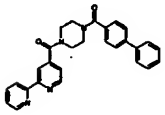
TM553



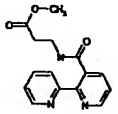
TM357



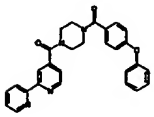
TM566



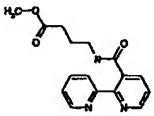
TM358



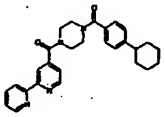
TM569



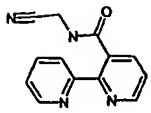
TM359



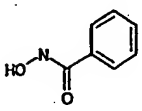
TM571



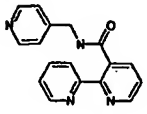
TM360



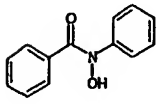
TM573



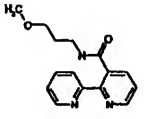
TM361



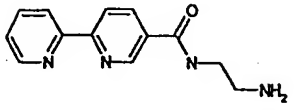
TM574



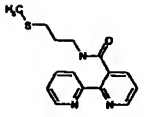
TM362



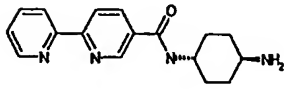
TM577



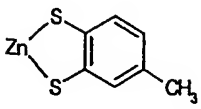
TM363



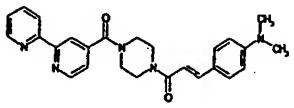
Examples of compounds used in examples herein



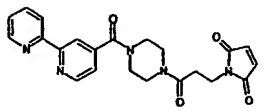
TM578



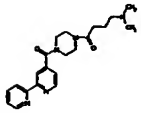
TM579



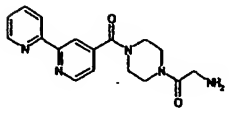
TM597



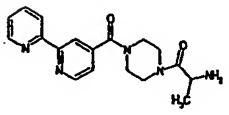
TM598



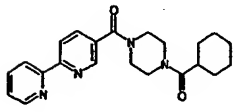
TM600



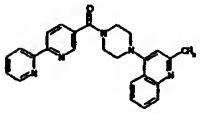
TM602



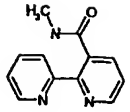
TM603



TM580



TM581



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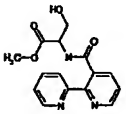
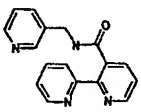
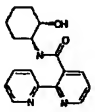


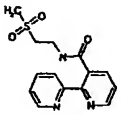
TM365



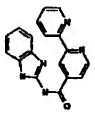
TM366



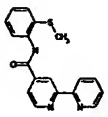
TM367



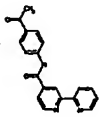
TM368



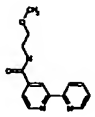
TM369



TM325

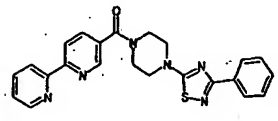


TM326

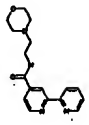


TM327

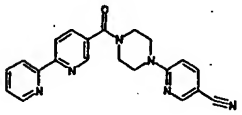
Examples of compounds used in examples herein



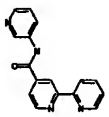
TM582



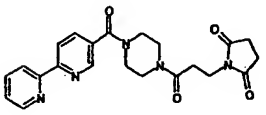
TM370



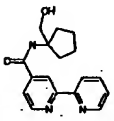
TM583



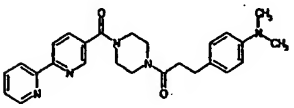
TM328



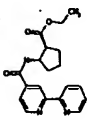
TM590



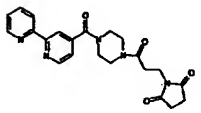
TM329



TM591



TM371



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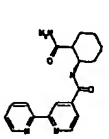
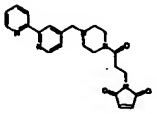
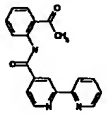


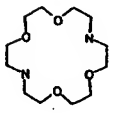
TM372



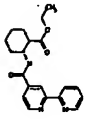
TM613



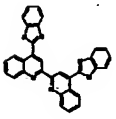
TM373



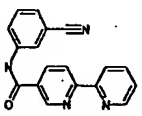
TM61



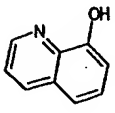
TM374



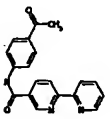
TM85



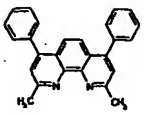
TM318



TM151



TM319



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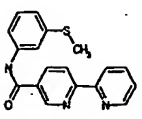
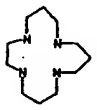
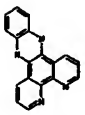


TM375

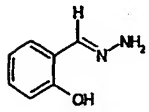
Examples of compounds used in examples herein



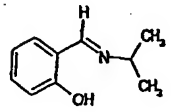
TM129



TM183



TM197



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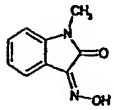
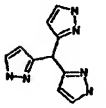
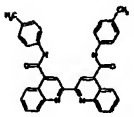


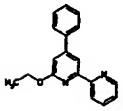
TM218



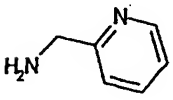
TM232



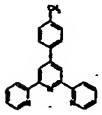
TM74



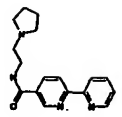
TM120



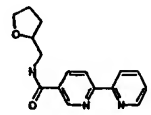
TM152



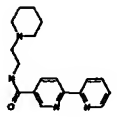
TM164



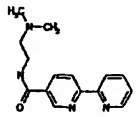
TM376



TM377



TM378



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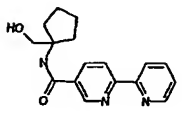

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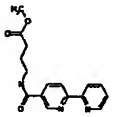
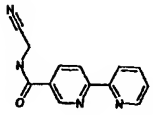


TM381



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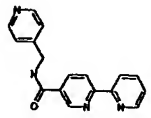
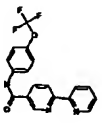


TM382



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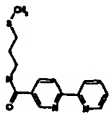
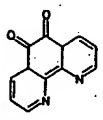
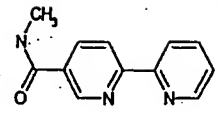


TM384

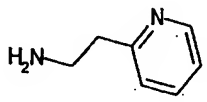
Examples of compounds used in examples herein



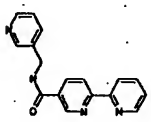
TM175



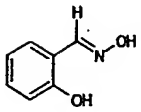
M385



TM184



TM386



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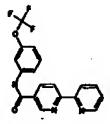
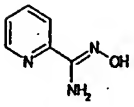
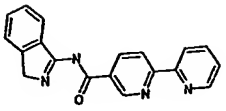


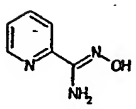
TM387



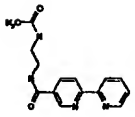
TM209



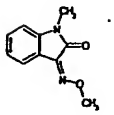
I321



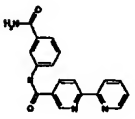
TM209



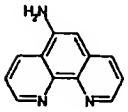
TM388



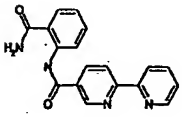
TM219



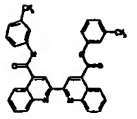
TM389



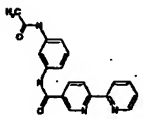
TM136



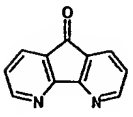
TM322



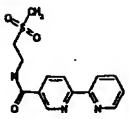
TM75



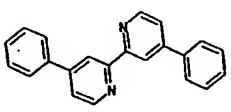
TM390



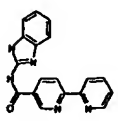
TM123



TM391

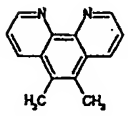


TM153

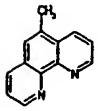


TM392

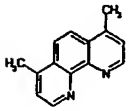
Examples of compounds used in examples herein



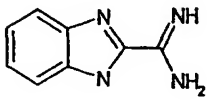
TM165



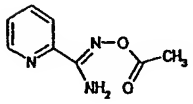
TM176



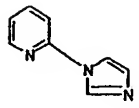
TM185



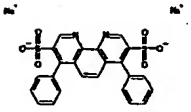
TM200



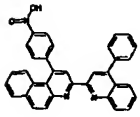
TM210



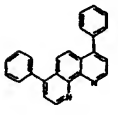
TM220



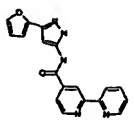
TM157



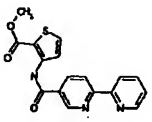
TM78



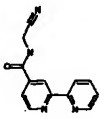
TM131



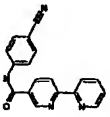
TM332



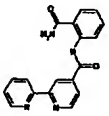
TM317



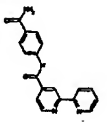
TM330



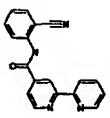
TM393



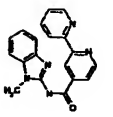
TM331



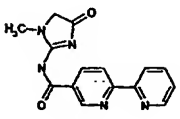
TM323



TM324

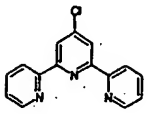


TM394

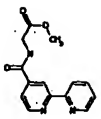


TM395

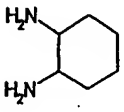
Examples of compounds used in examples herein



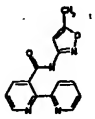
TM154



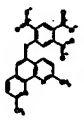
TM396



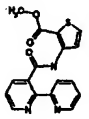
TM166



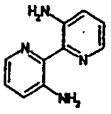
TM412



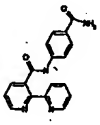
TM178



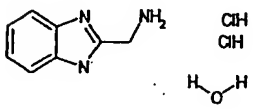
TM413



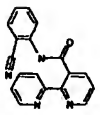
TM186



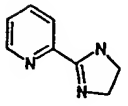
TM414



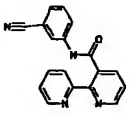
TM203



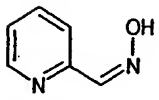
TM415



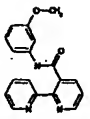
TM212



TM416



TM221



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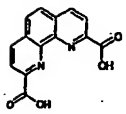
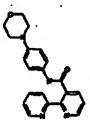
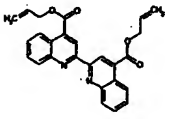


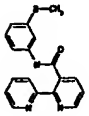
TM177



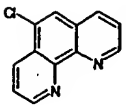
TM418



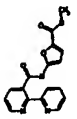
TM79



TM419

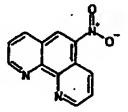


TM132

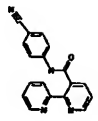


TM420

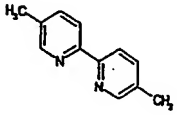
Examples of compounds used in examples herein



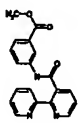
TM155



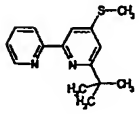
TM421



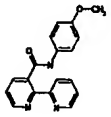
TM168



TM422



TM179



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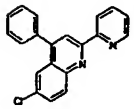
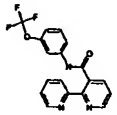


TM192



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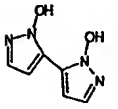
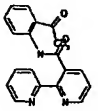


TM204



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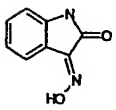
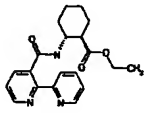
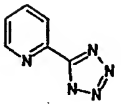


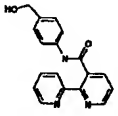
TM214



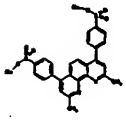
TM426



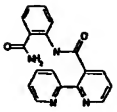
TM222



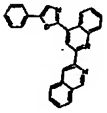
TM427



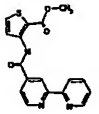
TM187



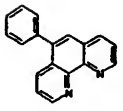
TM428



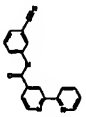
TM80



TM403

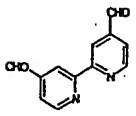


TM134

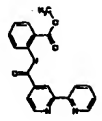


TM405

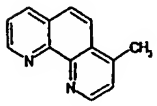
Examples of compounds used in examples herein



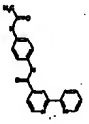
TM159



TM406



TM170



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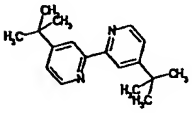

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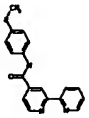
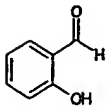
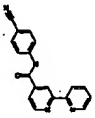


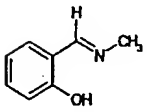
TM408



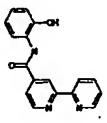
TM194



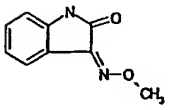
TM409



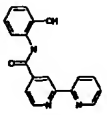
TM205



TM429



TM215



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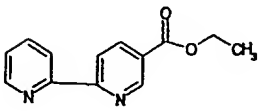

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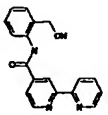

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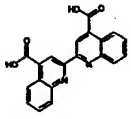
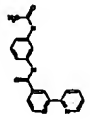
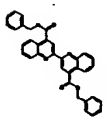


TM188



TM410



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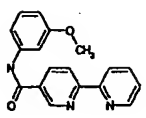

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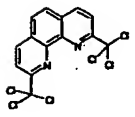
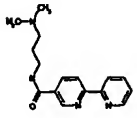


TM135



TM431

Examples of compounds used in examples herein

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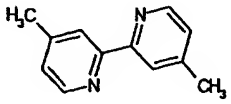
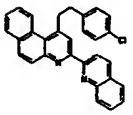


TM171



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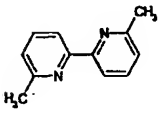

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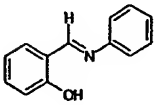

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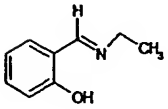
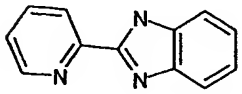


TM216



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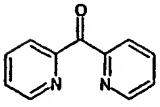

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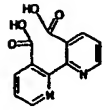
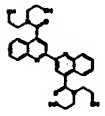
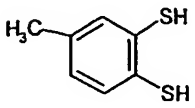


TM83



TM137



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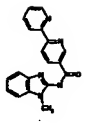
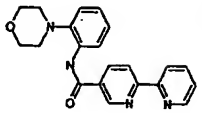
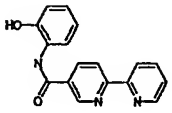


TM432



TM402



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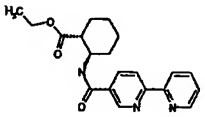

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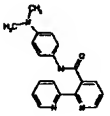
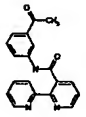
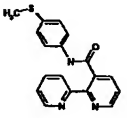


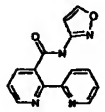
TM435



TM436



TM437



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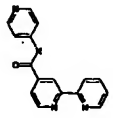

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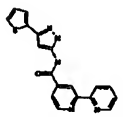
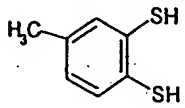
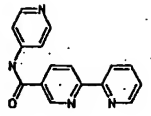


Examples of compounds used in examples herein



TM137



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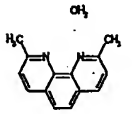
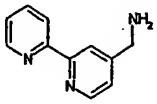


TM161



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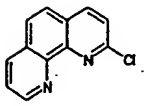

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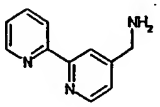

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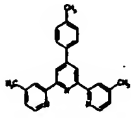

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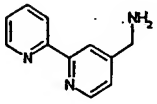
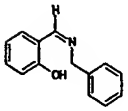


TM465



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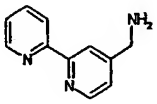

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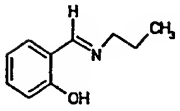

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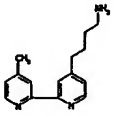

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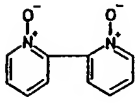

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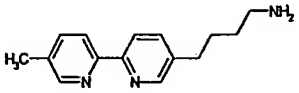

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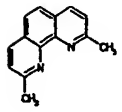
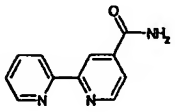
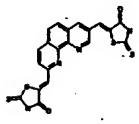


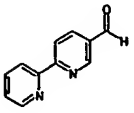
TM231



TM438



TM191



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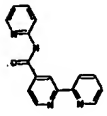
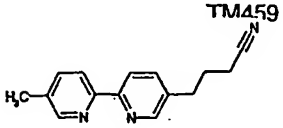


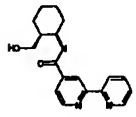
TM310



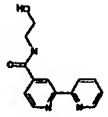
TM459

Examples of compounds used in examples herein

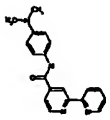
TM264



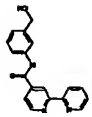
TM283



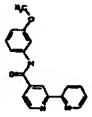
TM265



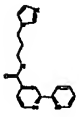
TM266



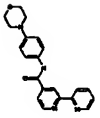
TM284



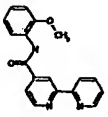
TM267



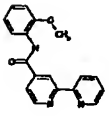
TM268



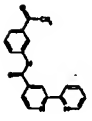
TM269



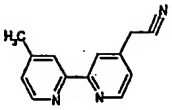
TM269



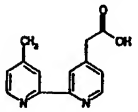
TM270

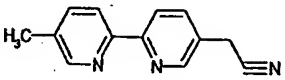


TM464

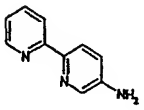


TM477

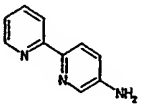




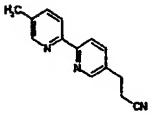
TM398



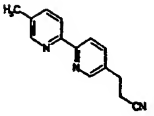
TM398



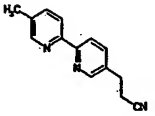
TM461



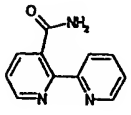
TM461



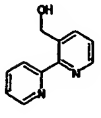
TM461



TM439

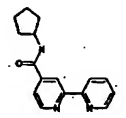


TM455

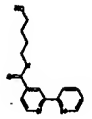


Examples of compounds used in examples herein

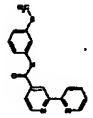
TM285



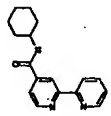
TM286



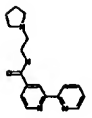
TM271



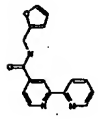
TM311



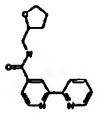
TM312



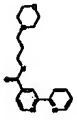
TM313



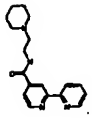
TM287



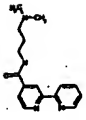
TM273



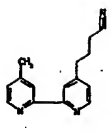
TM274



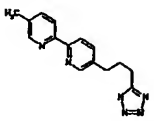
TM275



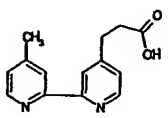
TM444



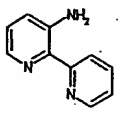
TM473



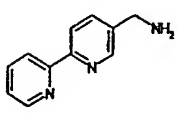
TM478



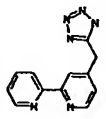
TM458



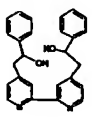
TM462



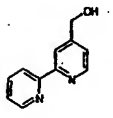
TM472



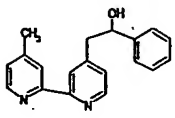
TM442



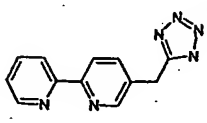
TM453



TM441

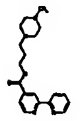


M475

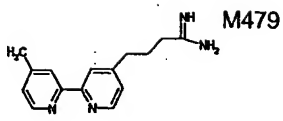


Examples of compounds used in examples herein

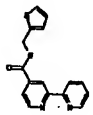
TM288



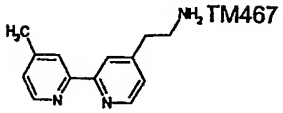
M479



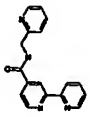
TM289

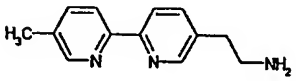


TM467

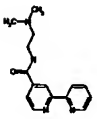


TM290

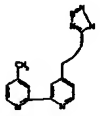




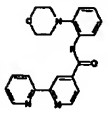
TM291



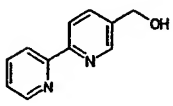
TM447



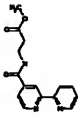
TM292



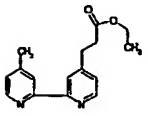
TM454



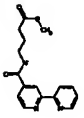
TM294



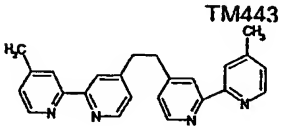
TM448



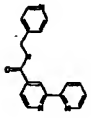
TM295



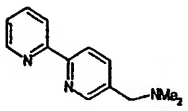
TM443



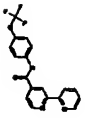
TM296



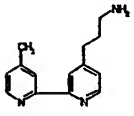
TM480



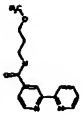
TM297



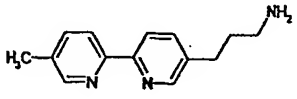
TM446



TM298

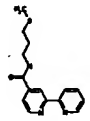


TM469

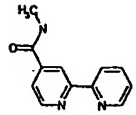


Examples of compounds used in examples herein

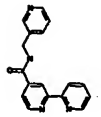
TM299



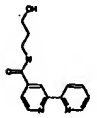
TM300



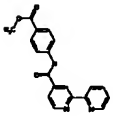
TM301



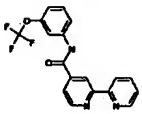
TM302



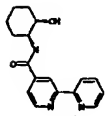
TM277



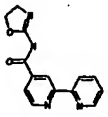
TM278



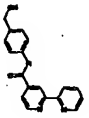
TM303



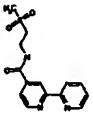
TM280



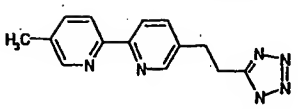
TM281



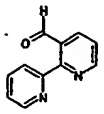
TM282



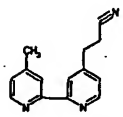
TM474



TM457



TM445



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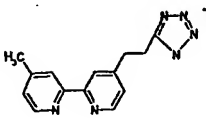
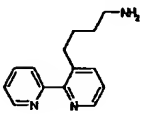
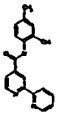


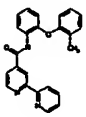
TM485



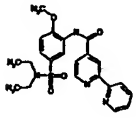
TM703



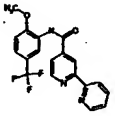
TM711



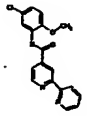
TM719



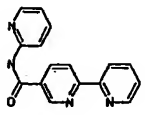
TM727



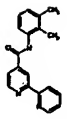
TM735



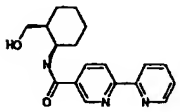
Examples of compounds used in examples herein



TM305



TM704



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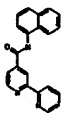

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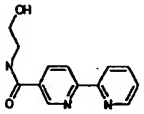
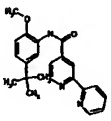


TM236



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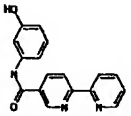
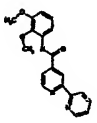
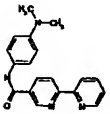


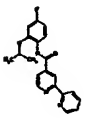
TM237



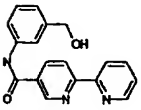
TM728



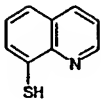
TM314



TM736



TM238



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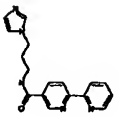

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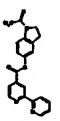

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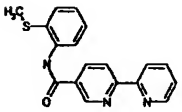
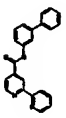
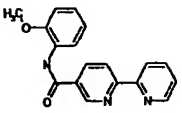


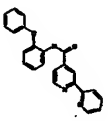
TM239



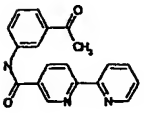
TM713



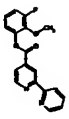
TM240



TM721

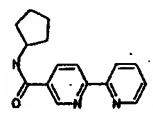


TM242

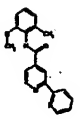


TM729

Examples of compounds used in examples herein



TM243



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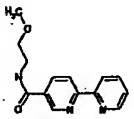
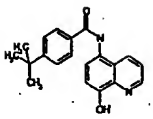
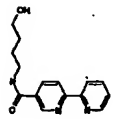


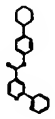
TM244



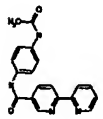
TM674



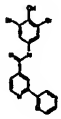
TM245



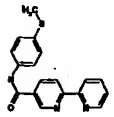
TM706



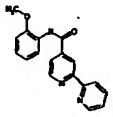
TM246



TM714



TM247



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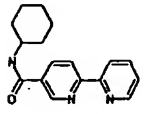
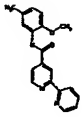
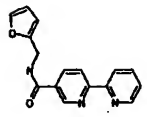


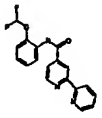
TM315



TM730



TM248



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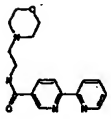

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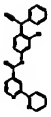

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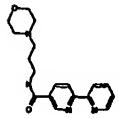

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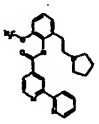

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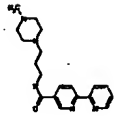

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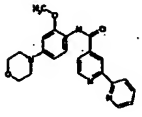
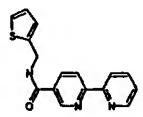


TM723

Examples of compounds used in examples herein



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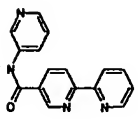

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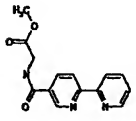

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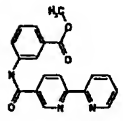

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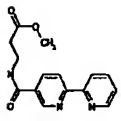

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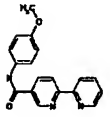

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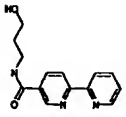

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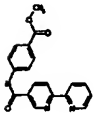

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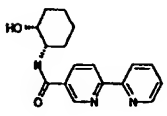

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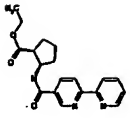

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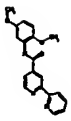

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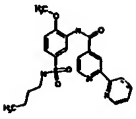

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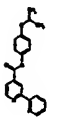

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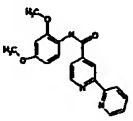

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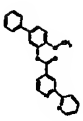

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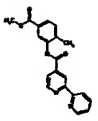

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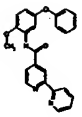

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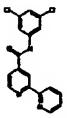

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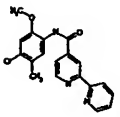

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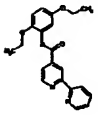
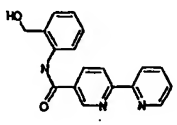


TM725

Examples of compounds used in examples herein



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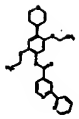

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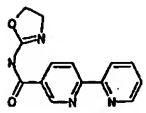

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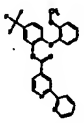

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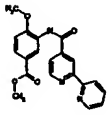

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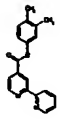

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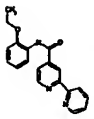

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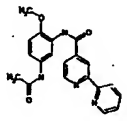

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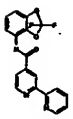
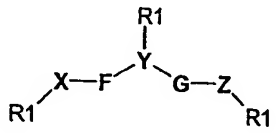


TM779

Claims

1. A library for use in a drug discovery process comprising three or more chemical compounds having at least two heteroatoms connected according to the general formula I abbreviated as Che-R^1



Formula I

wherein F is N, O, S, Se or P; and G is N, O, S, Se or P;

X, Y and Z, which are the same or different, are straight or branched C_1-C_{12} alkyl, C_1-C_{12} alkenyl, C_1-C_{12} alkynyl, C_1-C_{12} cyclyl, aryl, C_1-C_{12} heteroalkyl, C_1-C_{12} heteroalkenyl, C_1-C_{12} heteroalkynyl, C_1-C_{12} heterocyclyl, heteroaryl;

R^1 may be present anywhere on the X, Y and/or Z moiety and it may be present on X, Y and/or Z up to as many times as possible, i.e. if X is $-CH_2-CH_2-$, then R^1 may be present on the first and/or second carbon atom one or several times; R1 may optionally be hydrogen;

X may together with Y and/or Z fuse to form a cyclic ring system;
Y may together with X and/or Z fuse to form a cyclic ring system;
X, Y and Z may together fuse to form a cyclic ring system;

R^1 corresponds to a structure –A-B-C, wherein the element A is a coupling or connecting moiety, B is a spacer moiety and C is a functional group; –B- may be substituted one or more times with a further C, which may be the same or different, and

A linked to be –A-B-C is selected from the group consisting of:

-O-, -S-, -NH-, -N=, -N<, $-CH_2-$, -C(=O)-, $-PO_3-$, $-PO_2NH-$, $-NHPO_2$, -NHP(O)<, -C≡C-, -CH=CH-, -SO-, $-SO_2-$, -COO-, -CONR"-, -NR'CO-, $-NR'SO_2-$, $-SO_2NR"-$, -CH(OH)-, -CR'(OH)-, -CR'(O-alk)-, -N-alk-, aryl, cycloalkyl, heteroaryl, heterocycloalkyl etc., and the

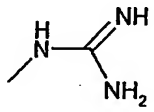
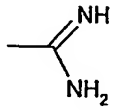
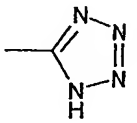
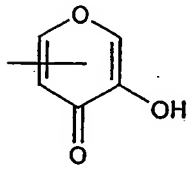
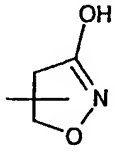
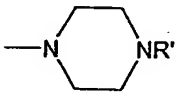
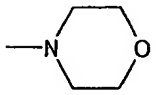
term "Alk" includes straight or branched alkyl, straight or branched alkenyl and straight or branched alkynyl; R' is H or lower alk, i.e. C_1-C_6; R" is as defined below;

-B- is absent or selected from the group consisting of:

H, alkyl, straight or branched alkyl, alkenyl (straight or branched), alkynyl (straight or branched), aryl, cycloalkyl, heteroaryl, heterocycloalkyl, alkyloxyalkyl, alkylaminoalkyl,

-C is absent or selected from the group consisting of:

-H, -OH, -NR"R'", -CONR"R'", -COOR", -OCOR", -COR", -SO_2NR"R'", -SH, -S-S-alk, -NHCOR", -NR"COR'", $NHSO_2R$", -$NHCONH_2$, -NH-CN, -F, -Cl, -Br, -I; -SCF_3, -CF_3, -OCF_3, -SCH_3, -SR", -CN, -$N(CN)_2$,-NO_2, -OCH_3, -OR', -NH_2, -NHMe, -NHAlk, -NMe_2, -$NAlk_2$, -NMeAlk, -$N(Alk)_3^+$, heteroaryl, heterocycloalkyl

and R" and/or R'" has the same meaning as given for B above optionally substituted with one or more C;

in those cases where a compound has two or more R^1 in positions adjacent to each other the –A- and/or –B- elements from the two individual R^1 may form a cyclic ring system;

in those cases where B is absent R^1 is –A-C or –A and in those cases where C is absent R^1 is –A-B or –A;

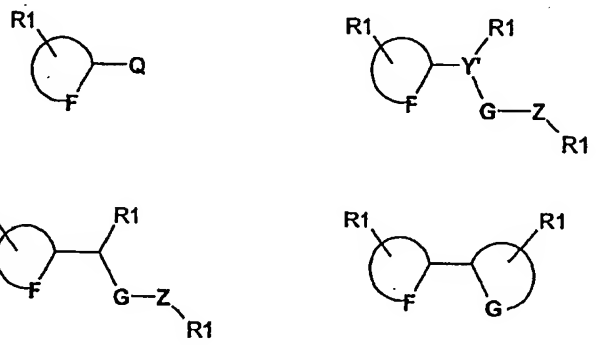
in some cases, A may be absent and then –R^1 is –B-C or –C, and B may be substituted one or more times with C, which may be the same or different;

the total number of atoms (X+F+Y+G+Z) excluding hydrogen atoms is at the most 25;

the total number of heteroatoms in (X+F+Y+G+Z) is at the most 6; and

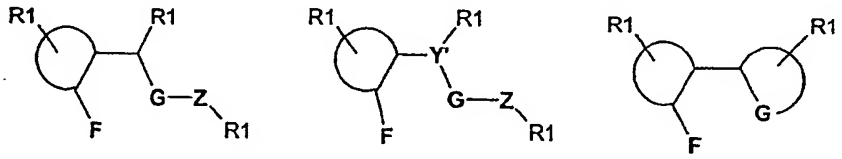
the size of a ring is at the most 14 atoms, preferably 5 or 6 atoms.

2. A library according to claim 1, wherein the compounds have one of the following structures



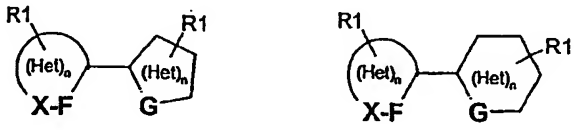
wherein Q is a structural element containing a heteroatom, Y' is the remainder of the group Y or it is absent, and F, G, Z and R^1 are as defined in formula I.

3. A library according to claim 1, wherein the compounds have one of the following structures



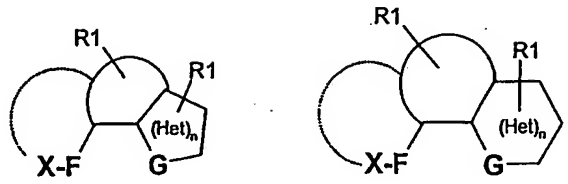
wherein F, G, Z, Y' and R1 are as defined in claims 1 or 2.

4. A library according to claim 1, wherein the compounds have one of the following structures



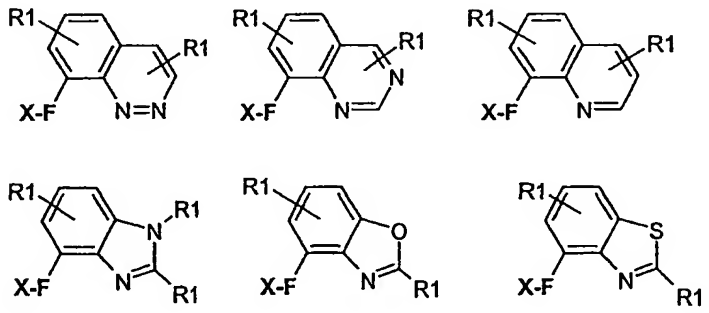
wherein the coordinating atom G is included in a 5-or 6-membered aromatic, unsaturated or saturated heterocycle containing between one and four heteroatoms and the coordinating atom F contained within an aromatic, unsaturated or saturated 5- or 6-memebered heterocycle containing between one and four heteroatoms.

5. A library according to claim 1, wherein the compounds have one of the following structures



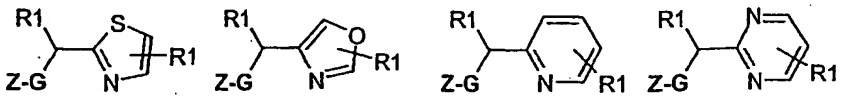
wherein the coordinating atom G is included in a 5- or 6-membered aromatic, unsaturated or saturated heterocycle containing between one and three heteroatoms and the coordinating atom F appended to an annelated aromatic, unsaturated or saturated 5- or 6-membered ring, and X-F can optionally be included in a fused ring as indicated by the dashed line.

6. A library according to claim 1, wherein the compounds have one of the following structures

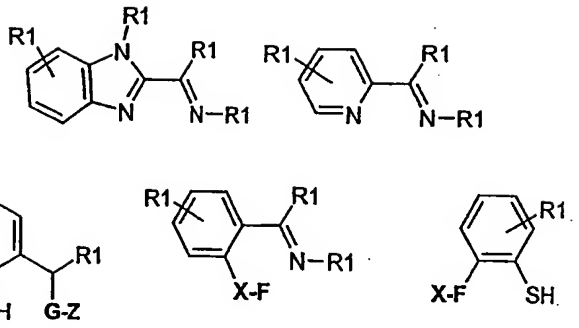


wherein R1 may be present on the annelated ring system to give mono-, di-, tri-, tetra-, pentasubstituted derivatives.

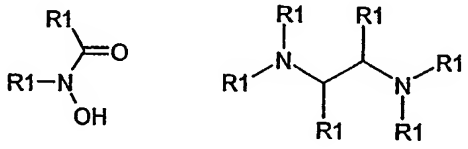
7. A library according to any of claims 1-3, wherein the compounds have one of the following structures



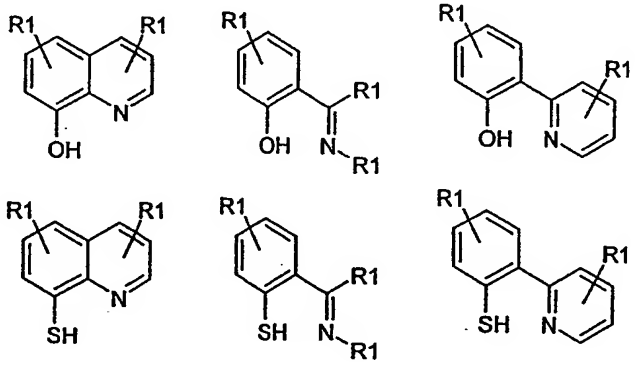
8. A library according to any of claims 1-3, wherein the compounds have one of the following structures



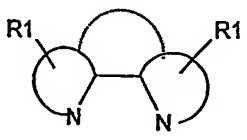
9. A library according to any of claims 1-3, wherein the compounds have one of the following structures



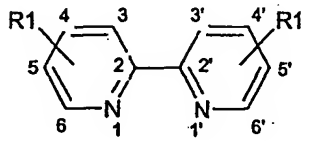
10. A library according to any of claims 1-3, wherein the compounds have one of the following structures



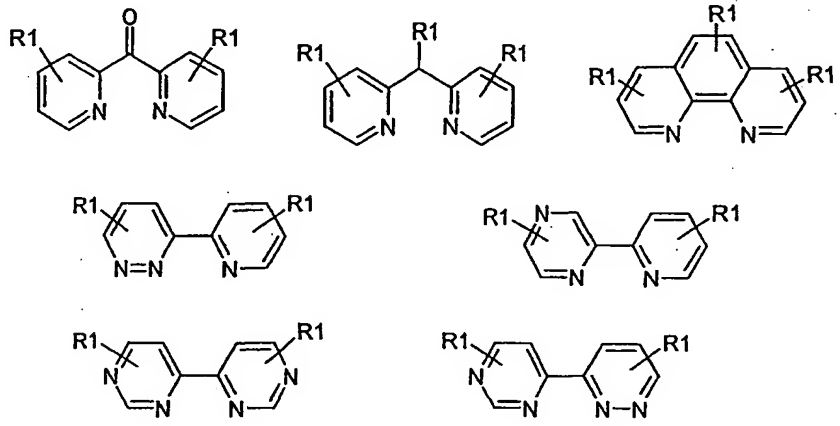
11. A library according to any of claims 1-3, wherein the compounds have one of the following structures



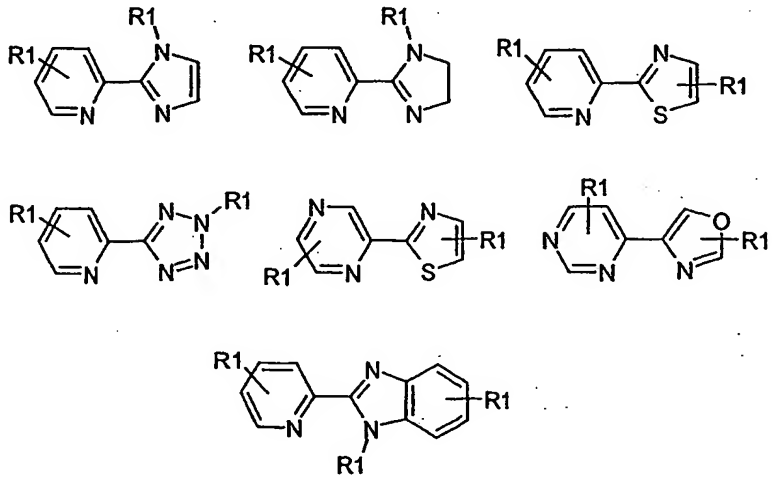
12. A library according to any of claims 1-3, wherein the compounds have one of the following structures



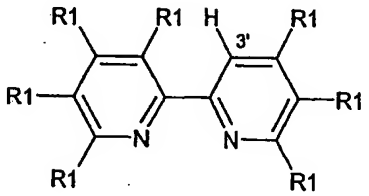
13. A library according to any of claims 1-3, wherein the compounds have one of the following structures



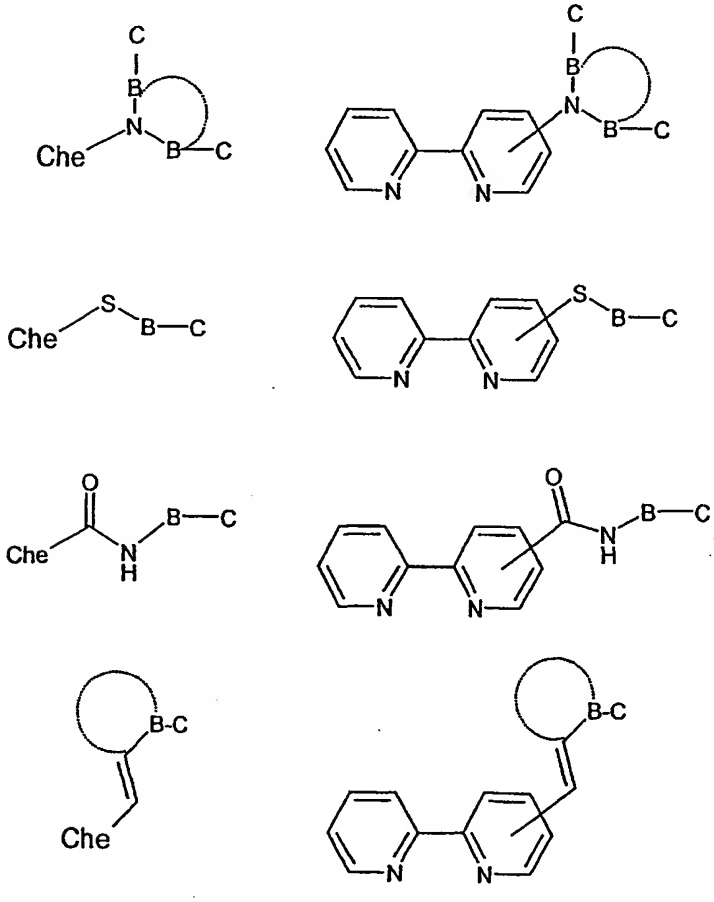
14. A library according to any of claims 1-3, wherein the compounds have one of the following structures



15. A library according to any of claims 1-3, wherein the compound has the following structure

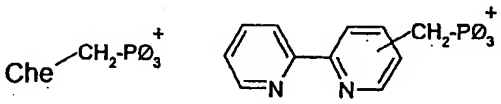


16. A library according to any of claims 1-15, wherein the compounds have one of the following structures Che-N(B-C)$_2$; Che-S-B-C ; Che-CO-NH-B-C ; Che-CH=CH-B-C ; Che-O-B-C ; Che-NH-CO-B-C ; Che-SO_2-NH-B-C, wherein Che represents a chelating scaffold according to any of claims 1-15.

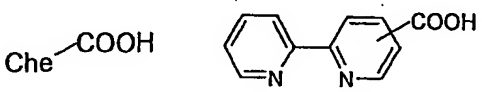


wherein B and C are as defined in formula I.

17. A library according to any of claims 1-15, wherein the library is an alkene-library with different B and/or C moieties comprising chemical compounds obtained by reacting appropriate ketone or aldehyde derivatives with ylides of phosphonium salts or phosphonates, wherein Ø means a phenyl group, such as

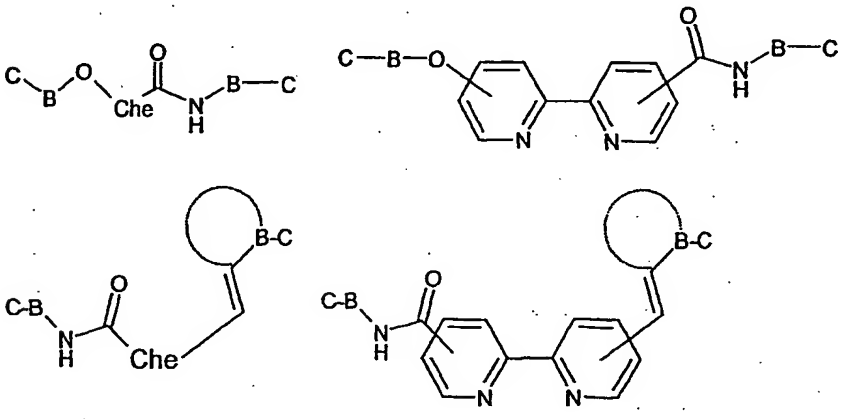


or an amide-library with different B and/or C moieties comprising chemical compounds obtained by reacting appropriate amine derivatives with carboxylic acid derivatives, in the presence of suitable coupling regents, such as



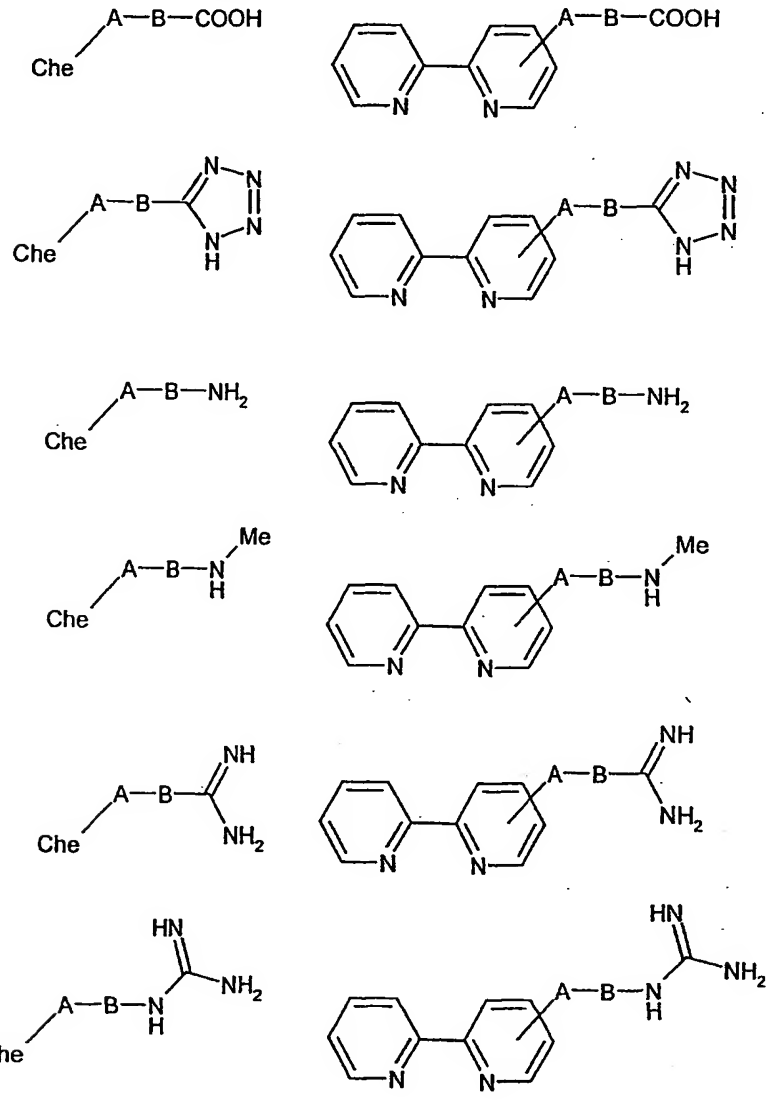
and wherein Che is a chelating scaffold according to any of claims 1-15.

18. A library according to any claims 1-15, wherein the library is an AA'-library comprising chemical compounds containing one or more of the R^1 groups shown for A-libraries in claim 15 in any combination as exemplified by the following structures C-B-O-Che-CO-NH-B-C ; C-B-NH-CO-Che-CH=CH-B-C ; C-B-NH-CO-Che-NH-CO-B-C ; C-B-NH-CO-Che-CO-NH-B-C wherein Che represents a chelating scaffold according to any of claims 1-15



and wherein B and C are as defined in formula I.

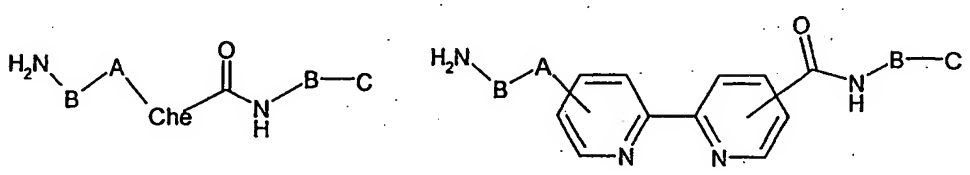
19. A library according to any claims 1-15, wherein the library is a C-library comprising chemical compounds of one or more of the following structures Che-A-B–CHO, Che-A-B-NH_2, Che-A-B-$NHCH_3$, Che-A-B-guanidin, Che-A-B-tetrazol, Che-A-B-COOH, Che-A-B-COONa, Che-A-B-$CONH_2$, Che-A-B-NO_2, Che-A-B-CN, wherein Che represents a chelating scaffold according to any of claims 1-15



and wherein A and B are as defined in formula I.

20. A library according to any of claims 1-15, wherein the library is an AC-library comprising chemical compounds as exemplified by the following structures Me_2N-B-A-

Che-NH-CO-B-C; HOOC-B-A-Che-CO-NH-B-C, wherein Che represents a chelating scaffold according to any of claims 1-15



and wherein A, B and C are as defined in formula I.

21. A library according to any of the preceding claims, wherein the chemical compounds form chelates with a metal ion.

22. A library according to claim 21, wherein the chemical compounds of formula I contained in the library are present in the form of chelates formed with one or more metal ions.

23. A library according to claim 21 or 22, wherein the metal ion is selected from the group consisting of aluminium, antimony, arsenic, astatine, barium, beryllium, bismuth, boron, cadmium, calcium, cerium, cesium, chromium, cobalt, copper, dysprosium, erbium, europium, gadolinium, gallium, germanium, gold, hafnium, holmium, indium, iridium, iron, lanthanum, lead, lutetium, magnesium, manganese, mercury, molybdenum, neodymium, nickel, niobium, osmium, palladium, platinum, polonium, praseodymium, promethium, rhenium, rhodium, rubidium, ruthenium, samarium, scandium, selenium, silicon, silver, strontium, tantalum, technetium, tellurium, terbium, thallium, thorium, thulium, tin, titanium, tungsten, vanadium, ytterbium, yttrium, zinc, zirconium, and oxidation states and isotopes thereof; in particular aluminium, antimony, barium, bismuth, calcium, chromium, cobalt, copper, europium, gadolinium, gallium, germanium, gold, indium, iron, lutetium, manganese, magnesium, nickel, osmium, palladium, platinum, rhenium, rhodium, rubidium, ruthenium, samarium, silver, strontium, technetium, terbium, thallium, thorium, tin, yttrium, zinc, and oxidation states or isotopes thereof; in particular calcium, cobalt, copper, europium, gadolinium, gallium, iron, magnesium, manganese, nickel, palladium, platinum, ruthenium, samarium, thallium, terbium and zink (and oxidation states or isotopes thereof, preferably cobalt (II, III), copper (I, II), nickel (II, III), zink (II) and platinum (0, II, V), palladium (0, II, IV), ruthenium (0, II, III, IV, VI, VIII) or isotopes thereof.

24. A library according to any of the preceding clams comprising

i) test compounds having the same chemical functional group, C,
ii) test compounds having the same or almost same spacer moiety, B, in order to establish a distance from the heteroatom containing skeleton to the chemical functional group; in such a library, the chemical functional group may be the same or different,
iii) test compounds which in principle are prepared by the same method and/or which have the same kind of attachment, A, to the basic common structural element,
iv) test compounds which are capable of establishing a non-covalent interaction,
v) different test compounds which are chelated with the same metal ion, and/or
vi) the same test compound, which is chelated with different metal ions.

25. A library according to any of the preceding claims in the form of a suitable container comprising a number of e.g. wells or tubes, each containing a chemical compound according to formula I, optionally in the form of a chelate.

26. A kit for use in a drug discovery process comprising a first and a second component, wherein the first component is a library according to any of claims 1-20 and the second component is a composition comprising a metal ion selected from the group of metal ions according to claim 23.

27. A kit according to claim 26, wherein the composition comprising a metal ion is in the form of a solution such as, e.g. an aqueous solution.

28. Use of a library or a kit according to any of the preceding claims in a drug discovery method according to any of items 1-40 herein.

29. Use of a kit according to claim 27, wherein the compounds of formula I contained in the kit substantially immediately before use is contacted with a solution of a metal ion selected from the group or metal ions according to claim 23 to form a chelate between the compound and the metal ion.

30. Use of a kit according to claim 26 or 27, wherein the compound employed is in the form of a chelate formed between the compound and a metal ion selected from the group consisting of cobalt (II, III), copper (I, II), nickel (II, III), zinc (II) and platinum (0, II, V), palladium (0, II, IV), ruthenium (0, II, III, IV, VI, VIII) or isotopes thereof.

31. Use of a compound according to formula I in a drug discovery method according to any of items 1-40 herein.

32. Use of a compound according to claim 31, wherein the compound substantially immediately before use is contacted with a solution of a metal ion selected from the group or metal ions according to claim 23 to form a chelate between the compound and the metal ion.

33. Use of a compound according to claim 31 or 32, wherein the compound employed is in the form of a chelate formed between the compound and a metal ion selected from the group consisting of cobalt (II, III), copper (I, II), nickel (II, III), zinc (II) and platinum (0, II, V), palladium (0, II, IV), ruthenium (0, II, III, IV, VI, VIII) or isotopes thereof.

34. A drug discovery process according to any of items 1-40 as described herein, wherein an amino acid residue defining the metal-ion binding site is in a position selected from the group consisting of

Single Positions:

transmembrane segment-I:
[I:01]
[I:03]
[I:04]
[I:06]
[I:07]
[I:10]
[I:11]
[I:14]
[I:17]
[I:18]
[I:21]
[I:22]

or in positions

transmembrane segment-II:
[II:05]
[II:06]
[II:07]

[II:09]
[II:10]
[II:13]
[II:14]
[II:17]
[II:18]
[II:20]
[II:21]
[II:22]
[II:24]
[II:25]
[II:26]

or in positions

transmembrane segment-III:

[III:04]
[III:05]
[III:08]
[III:09]
[III:11]
[III:12]
[III:13]
[III:15]
[III:16]
[III:17]
[III:18]
[III:19]
[III:20]
[III:21]
[III:22]
[III:23]
[III:24]
[III:25]
[III:26]
[III:27]
[III:28]
[III:29]
[III:30]

[III:32]

or in positions

transmembrane segment-IV:

[IV:01]

[IV:02]

[IV:05]

[IV:06]

[IV:09]

[IV:10]

[IV:12]

[IV:13]

[IV:14]

[IV:16]

[IV:17]

[IV:18]

[IV:19]

[IV:20]

or in positions

transmembrane segment-V:

[V:01]

[V:04]

[V:05]

[V:08]

[V:09]

[V:12]

[V:13]

[V:16]

[V:17]

[V:20]

[V:21]

[V:23]

[V:24]

[V:27]

[V:28]

or in positions

transmembrane segment-VI:

[VI:-06]

[VI:-04]
[VI:-03]
[VI:-02]
[VI:-01]
[VI:01]
[VI:02]
[VI:03]
[VI:05]
[VI:06]
[VI:08]
[VI:09]
[VI:12]
[VI:13]
[VI:16]
[VI:17]
[VI:19]
[VI:20]
[VI:21]
[VI:23]
[VI:24]

or in positions

transmembrane segment-VII:

[VII:02]
[VII:03]
[VII:05]
[VII:06]
[VII:07]
[VII:08]
[VII:09]
[VII:10]
[VII:11]
[VII:12]
[VII:13]
[VII:15]
[VII:16]
[VII:17]
[VII:19]

[VII:20]

35. A drug discovery process according to claim 34, wherein an amino acid residue defining the metal-ion binding site is in a position selected from the group consisting of

TM-I:

[I:03]

[I:07]

[I:10]

[I:14]

TM-II:

[II:17]

[II:18]

[II:20]

[II:21]

[II:24]

[II:25]

TM-III:

[III:04]

[III:05]

[III:08]

[III:09]

[III:19]

[III:23]

[III:27]

[III:29]

[III:32]

TM-IV:

[IV:12]

[IV:13]

[IV:16]

[IV:17]

[IV:19]

[IV:20]

TM-V:
[V:01]
[V:04]
[V:05]
[V:08]
[V:09]
[V:12]

TM-VI:
[VI:-01]
[VI:-02]
[VI:09]
[VI:16]
[VI:19]
[VI:20]
[VI:23]
[VI:24]

TM-VII:
[VII:02]
[VII:03]
[VII:06]
[VII:07]
[VII:10]
[VII:16]

36. A drug discovery process according to claim 34, wherein two of the amino acid residues defining the metal-ion binding site is in a position selected from the group consisting of

transmembrane segment-I:
[I:03;I:07]
[I:06;I:10]
[I:10;I:14]
[I:14;I:18]
[I:17;I:21]

[I:18;I:22]

or in positions

transmembrane segment-II:

[II:05;II:09]

[II:06;II:10]

[II:09;II:13]

[II:17;II:21]

[II:20;II:24]

[II:21;II:25]

or in positions

transmembrane segment-III:

[III:04;III:08]

[III:05;III:09]

[III:08;III:12]

[III:09;III:13]

[III:11;III:15]

[III:12;III:16]

[III:15;III:19]

[III:19;III:23]

[III:20;III:24]

[III:21;III:25]

[III:22;III:26]

[III:23;III:27]

[III:24;III:28]

or in positions

transmembrane segment-IV:

[IV:01;IV:05]

[IV:02;IV:06]

[IV:05;IV:09]

[IV:06;IV:10]

[IV:12;IV:16]

[IV:13;IV:17]

[IV:14;IV:18]

or in positions

transmembrane segment-V:

[V:01;V:05]

[V:04;V:08]
[V:05;V:09]
[V:08;V:12]
[V:09;V:13]
[V:12;V:16]
[V:13;V:17]
[V:16;V:20]
[V:17;V:21]
[V:20;V:24]
[V:23;V:27]
[V:24;V:28]

or in positions

transmembrane segment-VI:
[VI:-04;VI:01]
[VI:-03;VI:02]
[VI:-02;VI:03]
[VI:01;VI:05]
[VI:02;VI:06]
[VI:05;VI:09]
[VI:08;VI:12]
[VI:09;VI:13]
[VI:13;VI:17]
[VI:16;VI:20]
[VI:17;VI:21]
[VI:19;VI:23]
[VI:20;VI:24]

or in positions

transmembrane segment-VII:
[VII:02;VII:06]
[VII:03;VII:07]
[VII:06;VII:10]
[VII:07;VII:11]
[VII:09;VII:13]
[VII:12;VII:16]
[VII:13;VII:17]
[VII:15;VII:19]
[VII:16;VII:20]

or in positions

transmembrane segment-I and -II
[I:01;II:26]
[I:01;II:25]
[I:01;II:22]
[I:03;II:25]
[I:04;II:26]
[I:04;II:25]
[I:04;II:22]
[I:07;II:25]
[I:07;II:22]
[I:07;II:21]
[I:07;II:18]
[I:10;II:18]
[I:10;II:14]
[I:10;II:13]
[I:11;II:18]
[I:11;II:14]
[I:14;II:18]
[I:14;II:13]
[I:14;II:14]
[I:14;II:10]
[I:18;II:10]
[I:18;II:07]

or in positions

transmembrane segment-I and -VII
[I:03;VII:07]
[I:03;VII:03]
[I:07;VII:07]

or in positions

transmembrane segment-II and -III
[II:20;III:04]
[II:24;III:04]

or in positions

transmembrane segment-II and -VII
[II:17;VII:10]

[II:18;VII:10]
[II:21;VII:07]

or in positions

transmembrane segment-III and -IV
[III:05;IV:17]
[III:05;IV:20]
[III:09;IV:16]
[III:09;IV:17]

or in positions

transmembrane segment-III and –V
[III:05;V:01]
[III:05;V:04]
[III:05;V:08]
[III:09;V:01]
[III:09;V:04]
[III:09;V:08]
[III:12;V:08]
[III:13;V:08]
[III:13;V:12]
[III:16;V:12]
[III:16;V:16]
[III:17;V:16]
[III:19;V:16]
[III:20;V:16]
[III:23;V:27]
[III:23;V:23]
[III:26;V:20]
[III:26;V:24]
[III:26;V:27]
[III:27;V:23]
[III:27;V:27]
[III:30;V:23]
[III:30;V:27]

or in positions

transmembrane segment-III and -VI
[III:05;VI:16]

[III:08;VI:16]
[III:08;VI:13]
[III:09;VI:13]
[III:12;VI:13]
[III:13;VI:09]
[III:16;VI:09]
[III:19;VI:09]
[III:19;VI:06]
[III:19;VI:05]
[III:19;VI:09]
[III:22;VI:05]
[III:23;VI:09]
[III:25;VI:02]
[III:25;VI:-03]
[III:26;VI:02]
[III:26;VI:-03]
[III:26;VI:-02]
[III:26;VI:-06]
[III:27;VI:-01]
[III:27;VI:-02]
[III:29;VI:-02]
[III:30;VI:-06]
[III:30;VI:-02]
[III:32;VI:-02]

or in positions

transmembrane segment-III and -VII

[III:04;VII:07]
[III:05;VII:06]
[III:09;VII:09]
[III:08;VII:06]
[III:08;VII:10]
[III:11;VII:10]
[III:12;VII:06]
[III:12;VII:10]
[III:12;VII:12]
[III:15;VII:16]

[III:19;VII:16]

or in positions

transmembrane segment-IV and -V

[IV;19;V:04]
[IV;20;V:04]
[IV;12;V:12]
[IV;16;V:08]

or in positions

transmembrane segment-V and -VI

[V:01;VI:20]
[V:01;VI:24]
[V:05;VI:20]
[V:05;VI:24]
[V:09;VI:20]
[V:09;VI:21]
[V:09;VI:17]

or in positions

transmembrane segment-VI and -VII

[VI:23;VII:02]
[VI:23;VII:05]
[VI:20;VII:02]
[VI:19;VII:05]
[VI:19;VII:02]
[VI:16;VII:02]
[VI:16;VII:06]
[VI:16;VII:09]
[VI:16;VII:10]
[VI:12;VII:08]
[VI:12;VII:09]
[VI:09;VII:12]
[VI:09;VII:15]
[VI:05;VII:16]
[VI:05;VII:15]

37. A drug discovery process according to claim 34, wherein two of the amino acid residues defining the metal-ion binding site is in a position selected from the group consisting of

TM-I:
[I:03;I:07]
[I:10;I:14]

TM-II:
[II:17;II:21]
[II:20;II:24]
[II:21;II:25]

TM-III:
[III:04;III:08]
[III:05;III:09]

TM-IV:
[IV:12;IV:16]
[IV:13;IV:17]

TM-V:
[V:01;V:05]
[V:04;V:08]
[V:05;V:09]

TM-VI:
[VI:16;VI:20]
[VI:19;VI:23]
[VI:20;VI:24]

TM-VII:
[VII:02;VII:06]
[VII:03;VII:07]
[VII:06;VII:10]

TM-I / TM-VII

[I:03;VII:07]
[I:07;VII:07]
[I:03;VII:03]

TM-II / TM-III
[II:20;III:04]
[II:24;III:04]

TM-II / TM-VII
[II:17;VII:10]
[II:18;VII:10]
[II:21;VII:07]

TM-III / TM-IV
[III:05;IV:17]
[III:05;IV:20]
[III:09;IV:16]
[III:09;IV:17]

TM-III / TM-V
[III:05;V:01]

TM-III / TM-VI
[III:08;VI:16]
[III:19;VI:09]
[III:23;VI:09]
[III:27;VI:-01]
[III:27;VI:-02]
[III:29;VI:-02]
[III:32;VI:-02]

TM-III / TM-VII
[III:08;VII:06]
[III:19;VII:16]

TM-IV / TM-V
[IV;19;V:04]

[IV;20;V:04]
[IV;12;V:12]
[IV;16;V:08]

TM-V / TM-VI
[V:01;VI:20]
[V:01;VI:24]
[V:05;VI:20]
[V:05;VI:24]

TM-VI / TM-VII
[VI:23;VII:02]
[VI:23;VII:06]
[VI:19;VII:02]
[VI:19;VII:06]

38. A drug discovery process according to any of claims 34-37, wherein the amino acid residue in any of the positions claimed is selected from the group consisting of His, Cys, Asp, Glu, Trp, Tyr, Ser, Thr, Lys, Arg, Asn, Gln and Met.

39. A drug discovery process according to any of claims 34-38, wherein the amino acid residue in any of the positions claimed is selected from the group consisting of His, Cys, Asp and Glu.

40. A drug discovery process according claim 36 or 37, wherein the amino acid residues in any of the positions claimed is selected from the group consisting of

His-His
His-Cys
His-Asp
His-Glu
Cys-Cys
Cys-His
Cys-Asp
Cys-Glu
Asp-Asp
Asp-His

Asp-Cys
Asp-Glu
Glu-Glu
Glu-Asp
Glu-His
Glu-Cys

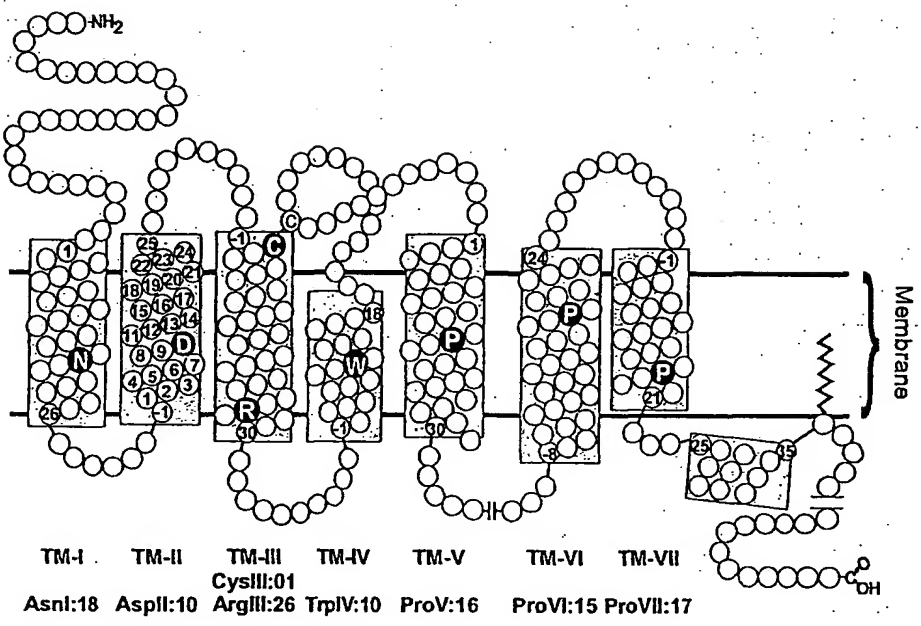

NH2
Membrane
TM-I
TM-II
TM-III
TM-IV
TM-V
TM-VI
TM-VII
CysIII:01
AsnI:18
AspII:10
ArgIII:26
TrpIV:10
ProV:16
ProVI:15
ProVII:17
OH

Fig. 1

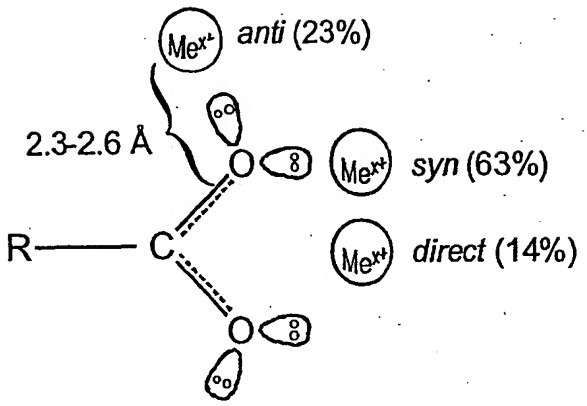


Fig. 2

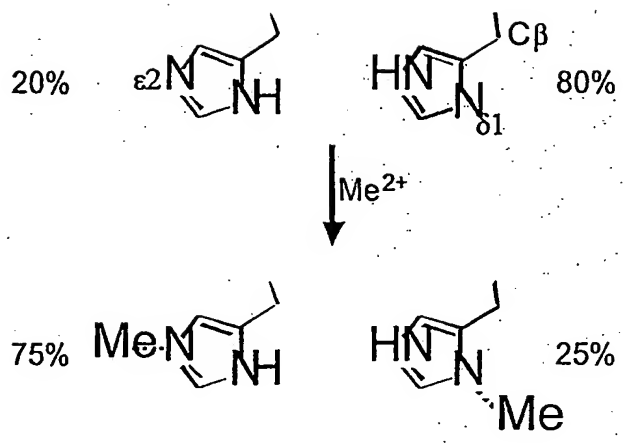


Fig. 3

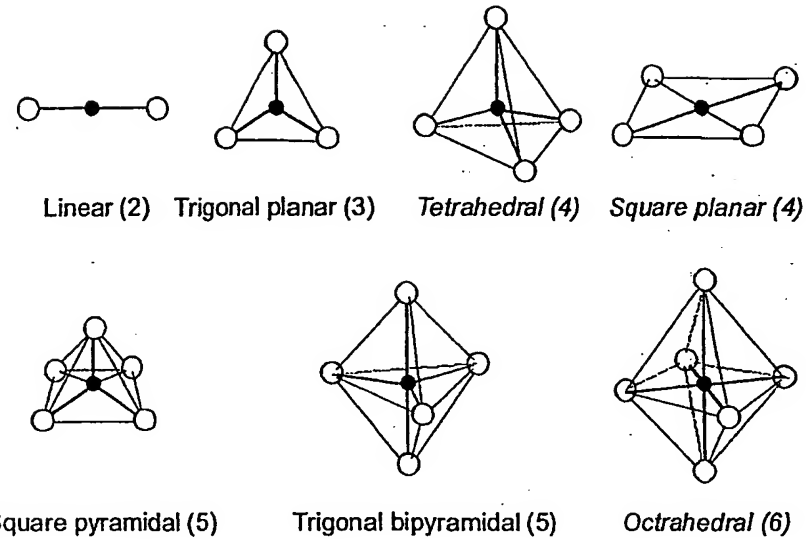

Linear (2)
Trigonal planar (3)
Tetrahedral (4)
Square planar (4)
Square pyramidal (5)
Trigonal bipyramidal (5)
Octrahedral (6)

Fig. 4

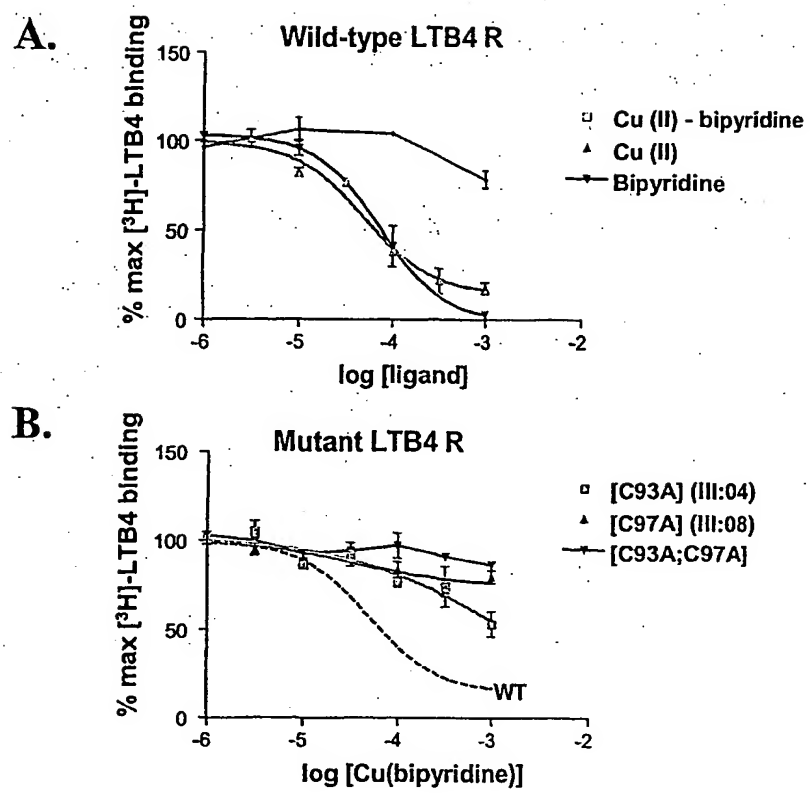
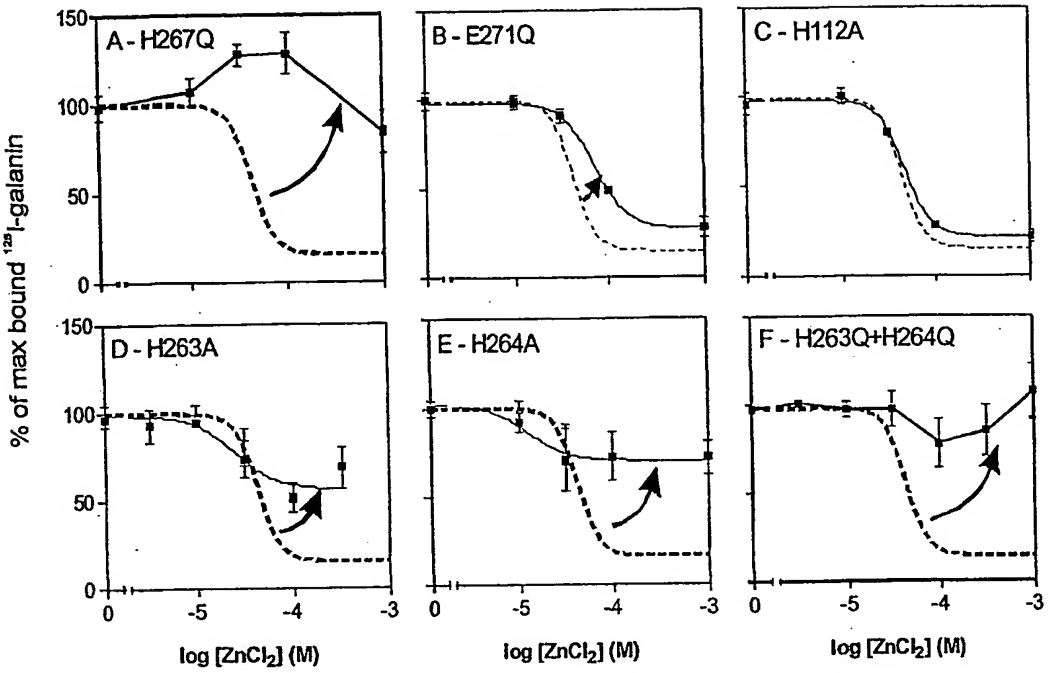

A.
Wild-type LTB4 R
% max [3H]-LTB4 binding
150
100
50
0
-6
-5
-4
-3
-2
log [ligand]
Cu (II) - bipyridine
Cu (II)
Bipyridine
B.
Mutant LTB4 R
% max [3H]-LTB4 binding
log [Cu(bipyridine)]
[C93A] (III:04)
[C97A] (III:08)
[C93A;C97A]
WT
C.
I.
II.
III.
IV.
V.
VI.
VII.

Fig. 5



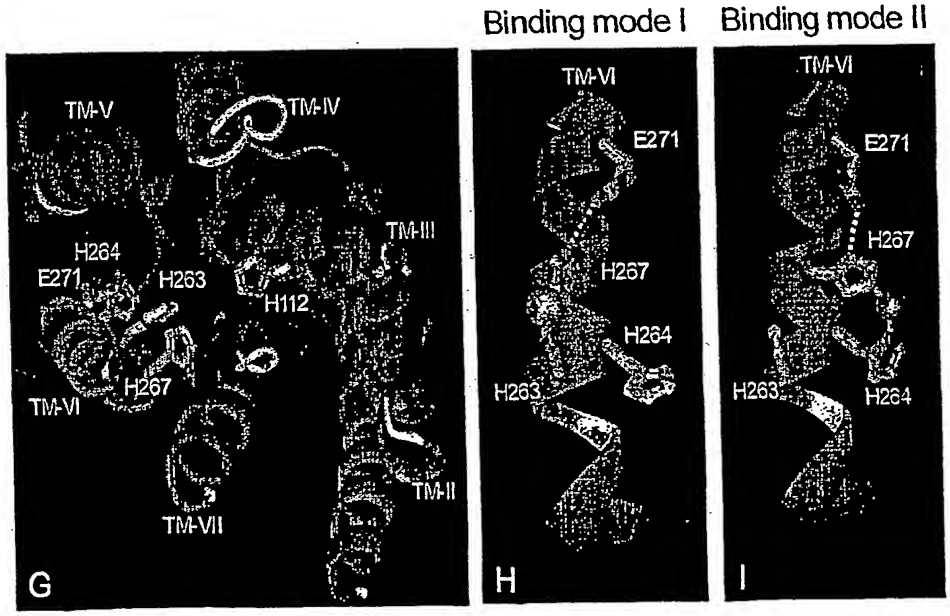
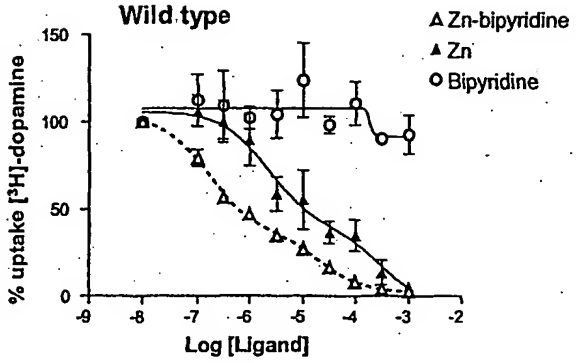
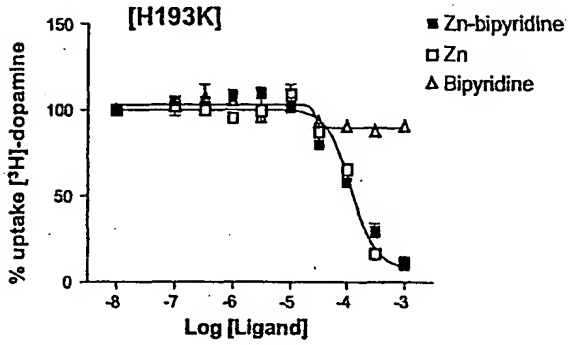


Fig. 6

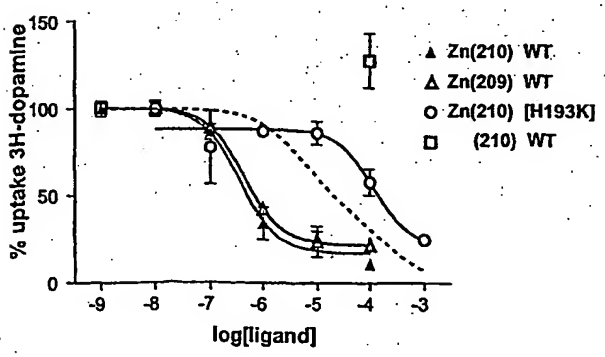
A.


Wild type
Zn-bipyridine
Zn
Bipyridine
% uptake [3H]-dopamine
Log [Ligand]

B.


[H193K]
Zn-bipyridine
Zn
Bipyridine
% uptake [3H]-dopamine
Log [Ligand]

C.


Zn(210) WT
Zn(209) WT
Zn(210) [H193K]
(210) WT
% uptake 3H-dopamine
log[ligand]

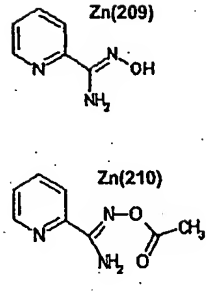
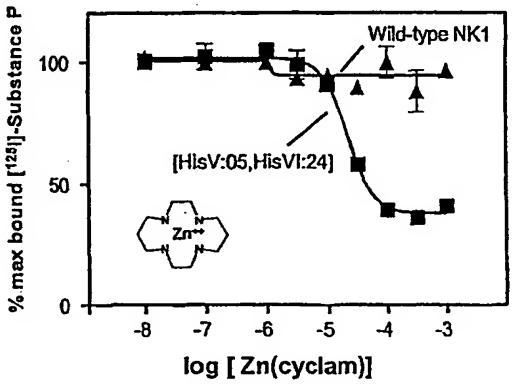

Zn(209)
Zn(210)

Fig. 7

A.

		Ions				1,10-Phenanthroline-complexes				2,2-Bipyridine-complexes			
		Zn	±SEM	(n)	Cu ±SEM (n)	Zn	±SEM	(n)	Cu ±SEM (n)	Zn	±SEM	(n)	Cu ±SEM (n)
WT hNK1		320	±20	(5)	370 ± 30 (5)	490	±50	(4)	480 ± 60 (3)	390	±60	(4)	150 ± 30 (2)
Y92H	II:24;III:04	17	± 3	(3)	28 ± 5 (2)	26	± 5	(2)	28 ± 6 (3)	31	± 5	(3)	25 ± 3 (2)
E193H;N109H	III:05;V:01	13	± 5	(2)	120 ± 20 (2)	46	± 9	(2)	120 ± 20 (2)	13	± 4	(2)	160 ± 30 (2)
P112H;M291C	III:08;VII:06	41	± 9	(6)	82 ± 15 (2)	63	± 4	(5)	45 ± 12 (4)	21	± 2	(4)	13 ± 2 (4)
Y272H	V:05;VI:24	9.1	± 1	(3)	330 ± 50 (3)	6.8	± 1.2	(3)	150 ± 20 (2)	9.8	± 3.2	(2)	140 ± 20 (2)

B.



C.

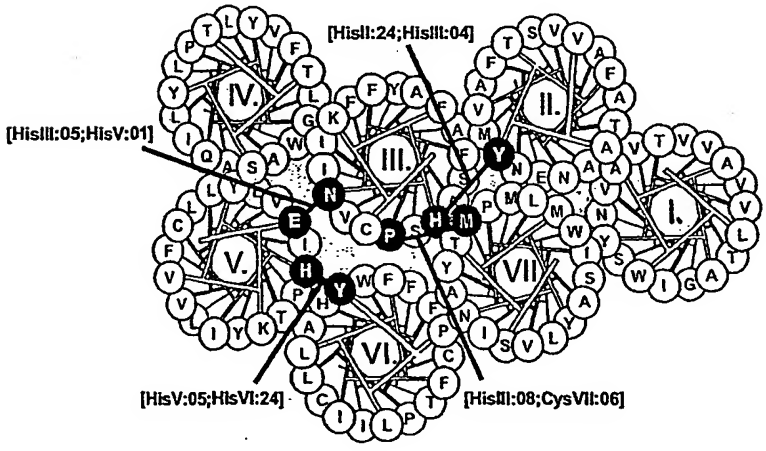
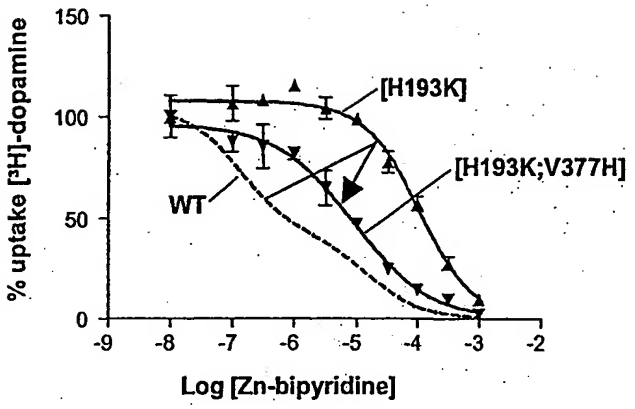


Fig. 8


% uptake [3H]-dopamine
150
100
50
0
-9
-8
-7
-6
-5
-4
-3
-2
Log [Zn-bipyridine]
[H193K]
[H193K;V377H]
WT

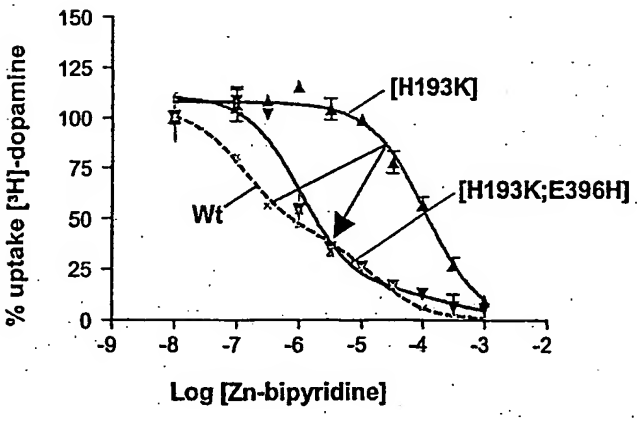

% uptake [3H]-dopamine
150
125
100
75
50
25
0
-9
-8
-7
-6
-5
-4
-3
-2
Log [Zn-bipyridine]
[H193K]
[H193K;E396H]
Wt

Fig. 9

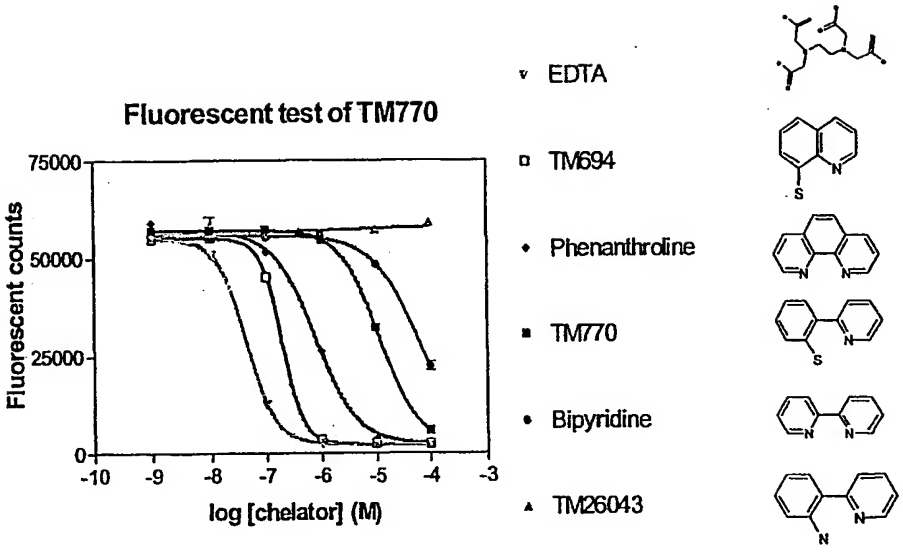

Fluorescent test of TM770
Fluorescent counts
75000
50000
25000
0
-10
-9
-8
-7
-6
-5
-4
-3
log [chelator] (M)
EDTA
TM694
Phenanthroline
TM770
Bipyridine
TM26043

Fig. 10

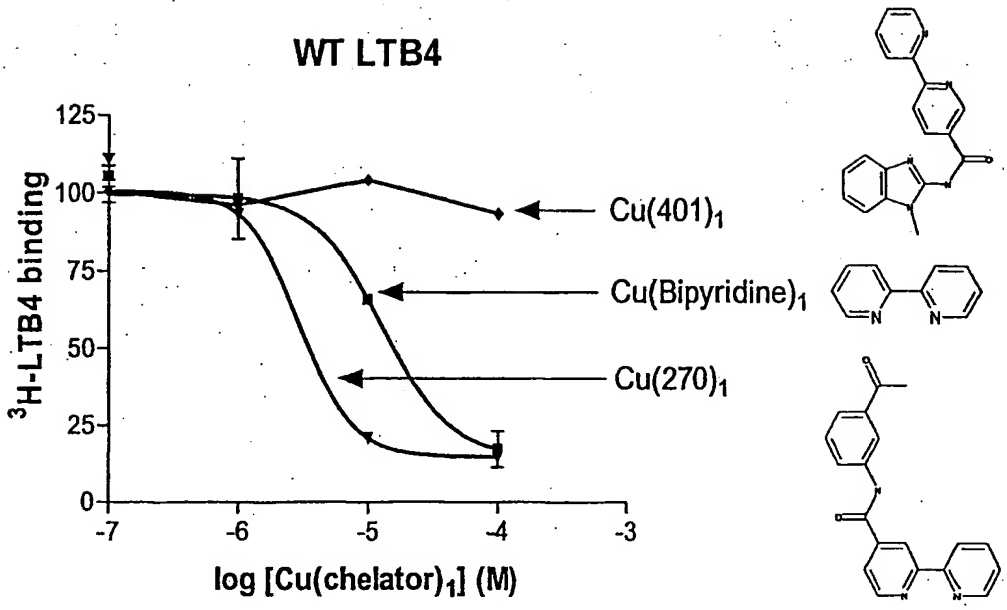
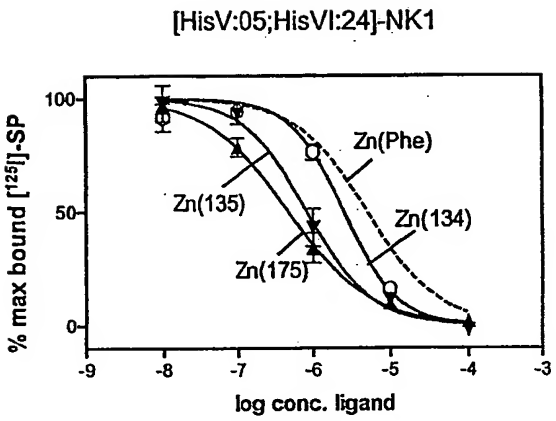
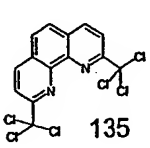
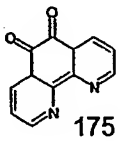

WT LTB4
125
100
75
50
25
0
-7
-6
-5
-4
-3
^{3}H-LTB4 binding
log [Cu(chelator)$_1$] (M)
Cu(401)$_1$
Cu(Bipyridine)$_1$
Cu(270)$_1$

Fig. 11

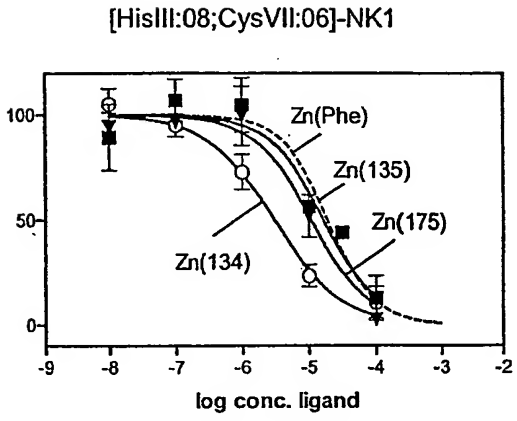
A.


[HisV:05;HisVI:24]-NK1
% max bound [125I]-SP
100
50
0
-9
-8
-7
-6
-5
-4
-3
log conc. ligand
Zn(Phe)
Zn(135)
Zn(134)
Zn(175)


135


175

B.


[HisIII:08;CysVII:06]-NK1
100
50
0
-9
-8
-7
-6
-5
-4
-3
-2
log conc. ligand
Zn(Phe)
Zn(135)
Zn(175)
Zn(134)

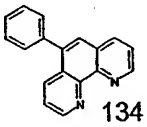
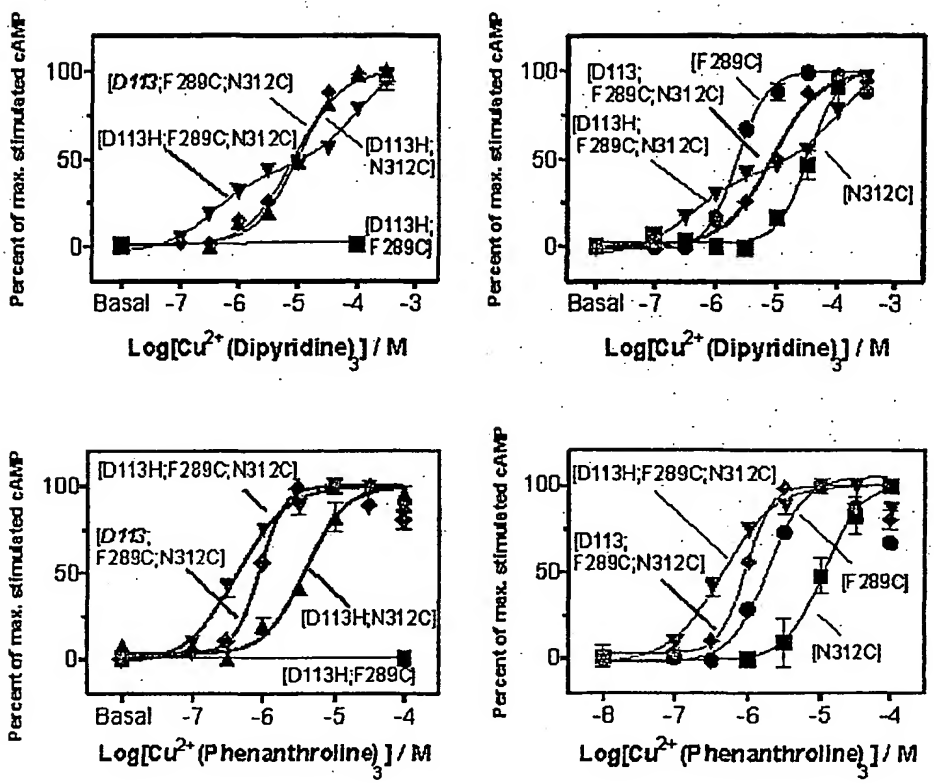

134

Fig. 12

A.


Percent of max. stimulated cAMP
100
50
0
[D113;F289C;N312C]
[D113H;F289C;N312C]
[D113H;N312C]
[D113H;F289C]
Basal -7 -6 -5 -4 -3
Log[Cu2+(Dipyridine)3] / M
[D113;F289C;N312C]
[F289C]
[D113H;F289C;N312C]
[N312C]
[D113H;F289C;N312C]
[D113;F289C;N312C]
[D113H;N312C]
[D113H;F289C]
Basal -7 -6 -5 -4
Log[Cu2+(Phenanthroline)3] / M
[D113H;F289C;N312C]
[D113;F289C;N312C]
[F289C]
[N312C]
-8 -7 -6 -5 -4
Log[Cu2+(Phenanthroline)3] / M

B.

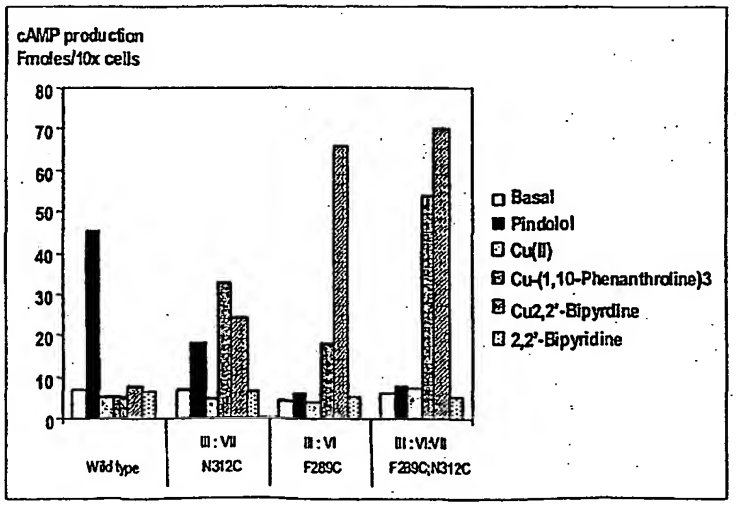
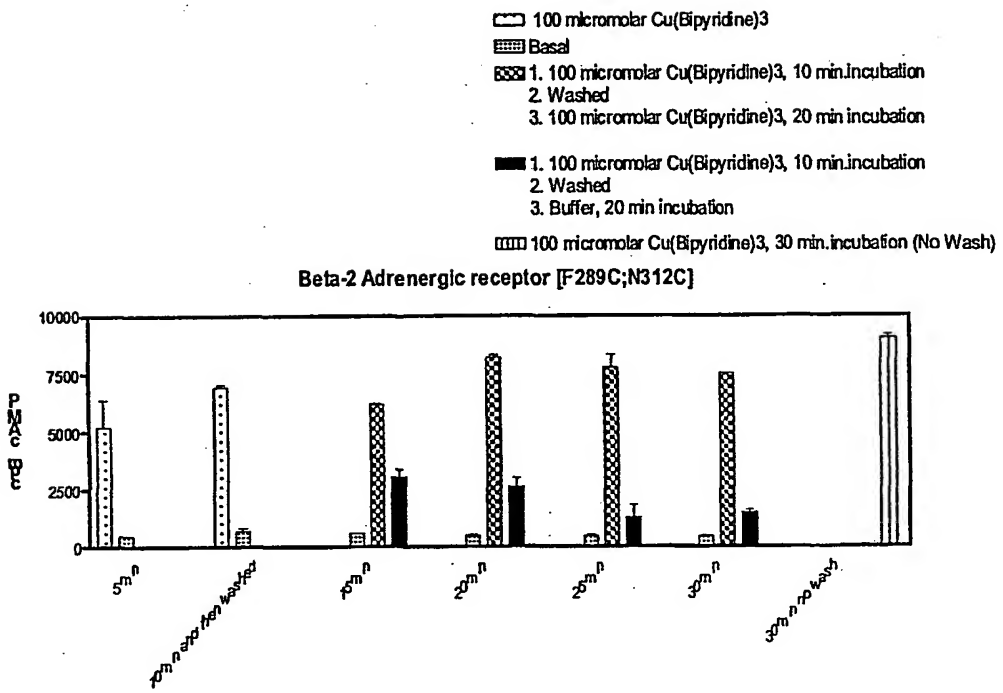

cAMP production
Fmoles/10x cells
80
70
60
50
40
30
20
10
0
Basal
Pindolol
Cu(II)
Cu-(1,10-Phenanthroline)3
Cu2,2'-Bipyridine
2,2'-Bipyridine
Wild type
III : VII N312C
III : VI F289C
III : VI:VII F289C;N312C

Fig. 13

A.


100 micromolar Cu(Bipyridine)3
Basal
1. 100 micromolar Cu(Bipyridine)3, 10 min.Incubation
2. Washed
3. 100 micromolar Cu(Bipyridine)3, 20 min incubation
1. 100 micromolar Cu(Bipyridine)3, 10 min.incubation
2. Washed
3. Buffer, 20 min incubation
100 micromolar Cu(Bipyridine)3, 30 min.incubation (No Wash)
Beta-2 Adrenergic receptor [F289C;N312C]
10000
7500
5000
2500
0
5min
10min after then washed
15min
20min
25min
30min
30min no wash

B.

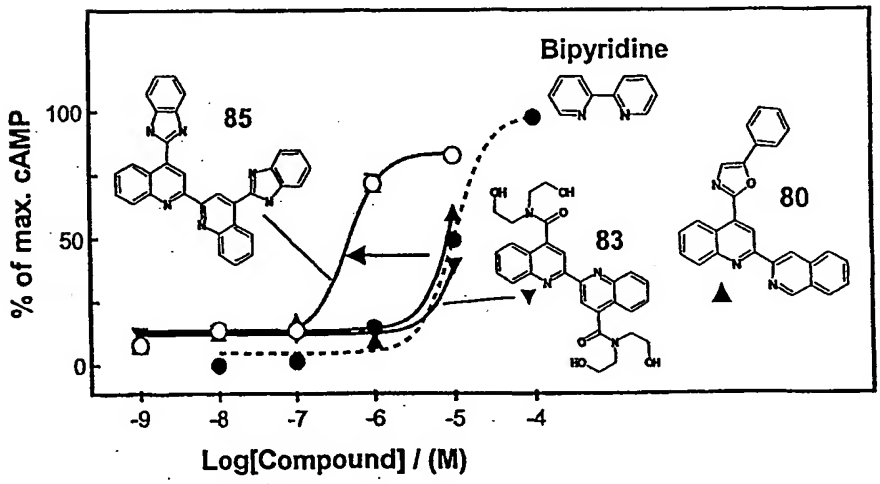

Bipyridine
85
83
80
100
50
0
% of max. cAMP
-9
-8
-7
-6
-5
-4
Log[Compound] / (M)

Fig. 14

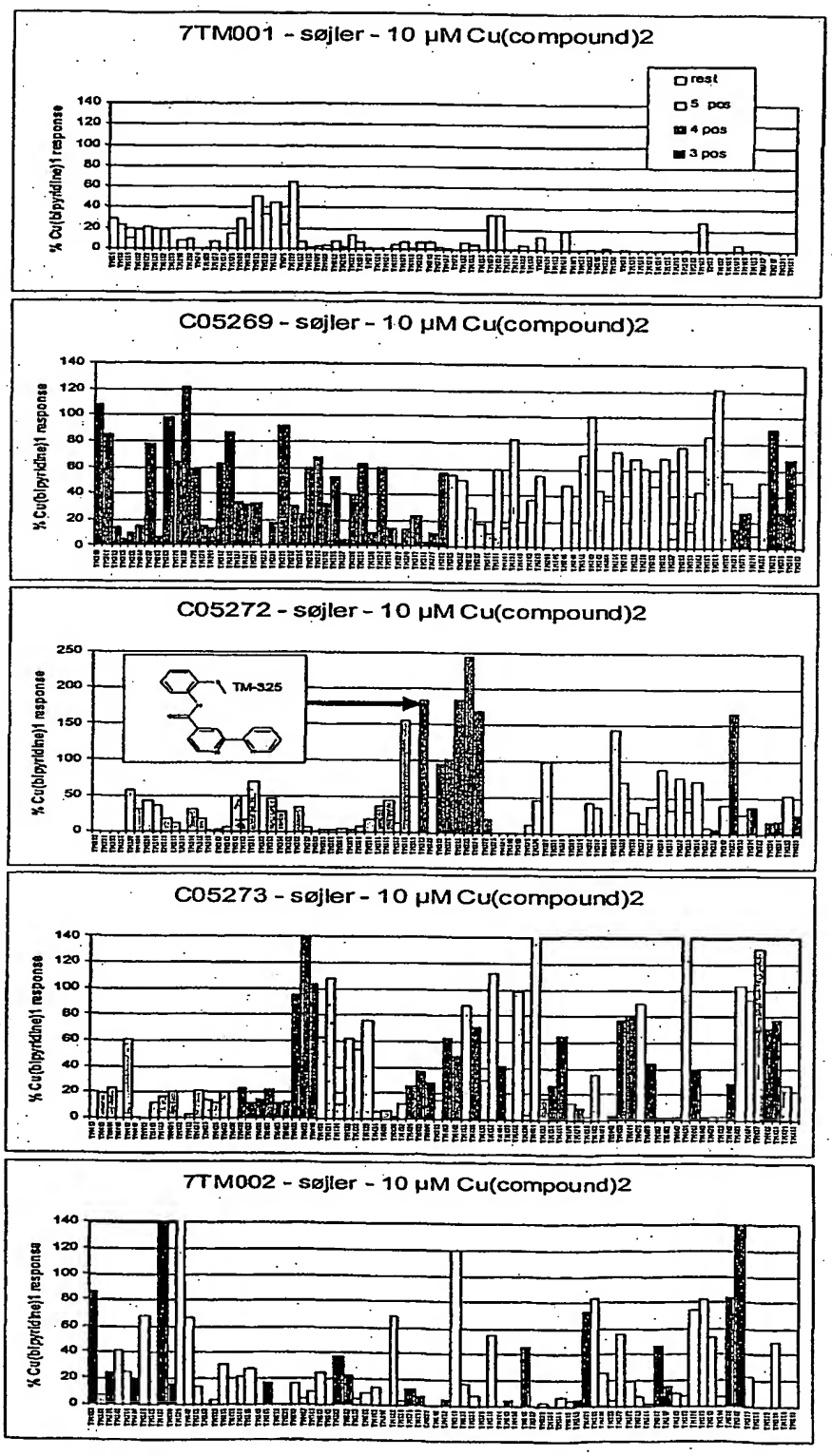

7TM001 - søjler - 10 µM Cu(compound)2
rest
5 pos
4 pos
3 pos
% Cu(bipyridine)1 response
C05269 - søjler - 10 µM Cu(compound)2
% Cu(bipyridine)1 response
C05272 - søjler - 10 µM Cu(compound)2
TM-325
% Cu(bipyridine)1 response
C05273 - søjler - 10 µM Cu(compound)2
% Cu(bipyridine)1 response
7TM002 - søjler - 10 µM Cu(compound)2
% Cu(bipyridine)1 response

Fig. 15

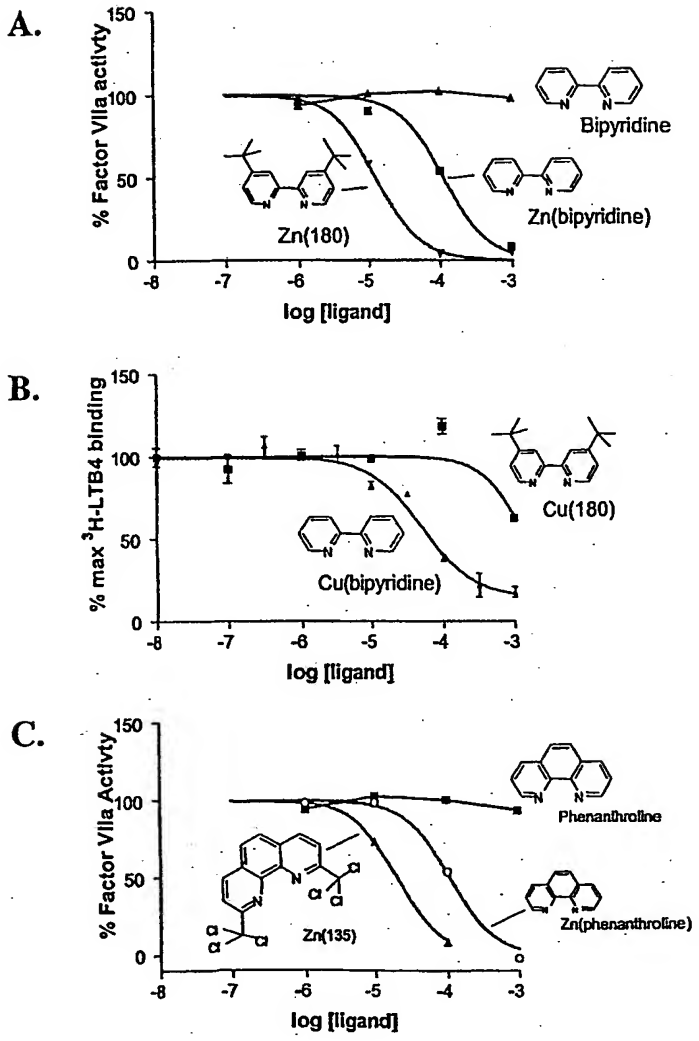

A.
% Factor VIIa activty
150
100
50
0
-8
-7
-6
-5
-4
-3
log [ligand]
Bipyridine
Zn(bipyridine)
Zn(180)
B.
% max ^{3}H-LTB4 binding
Cu(180)
Cu(bipyridine)
C.
% Factor VIIa Activty
Phenanthroline
Zn(phenanthroline)
Zn(135)

Fig. 16

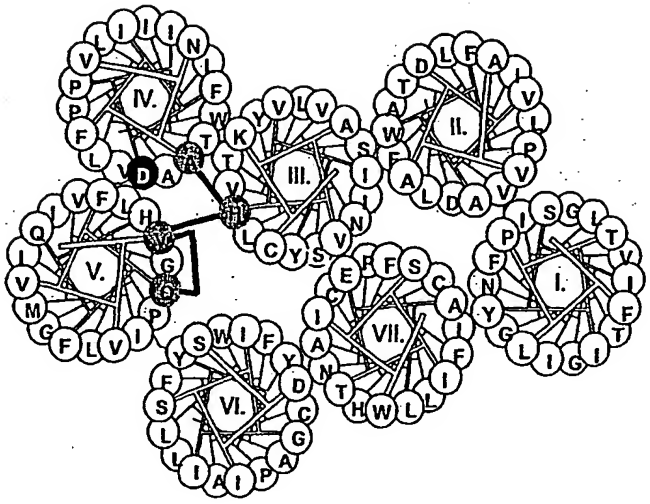
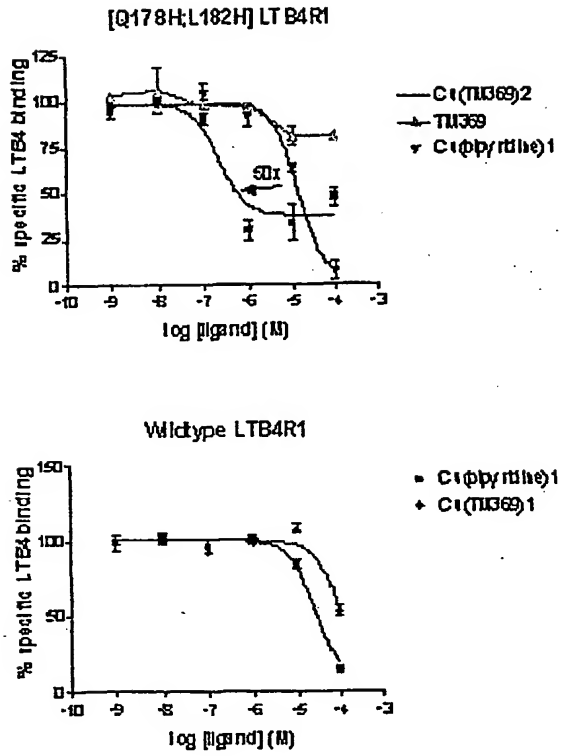
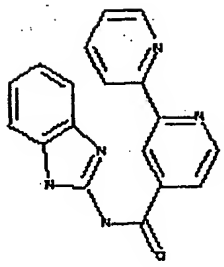


Fig. 17


[Q178H;L182H] LTB4R1
% specific LTB4 binding
log [ligand] (M)
Ct(TM369)2
TM369
Ct(pyrithione)1
50x
Wildtype LTB4R1
% specific LTB4 binding
log [ligand] (M)
Ct(pyrithione)1
Ct(TM369)1



TM-369

Fig. 18

Metal-ion	IC50 on rKAPPA BisHis receptor
Zn(II)	1.1 µM
Co(II)	2.1 µM
Ni(II)	2.7 µM
Cu(II)	4.0 µM
Gd(III)	42 µM
Mn(II)	57 µM

Fig. 19

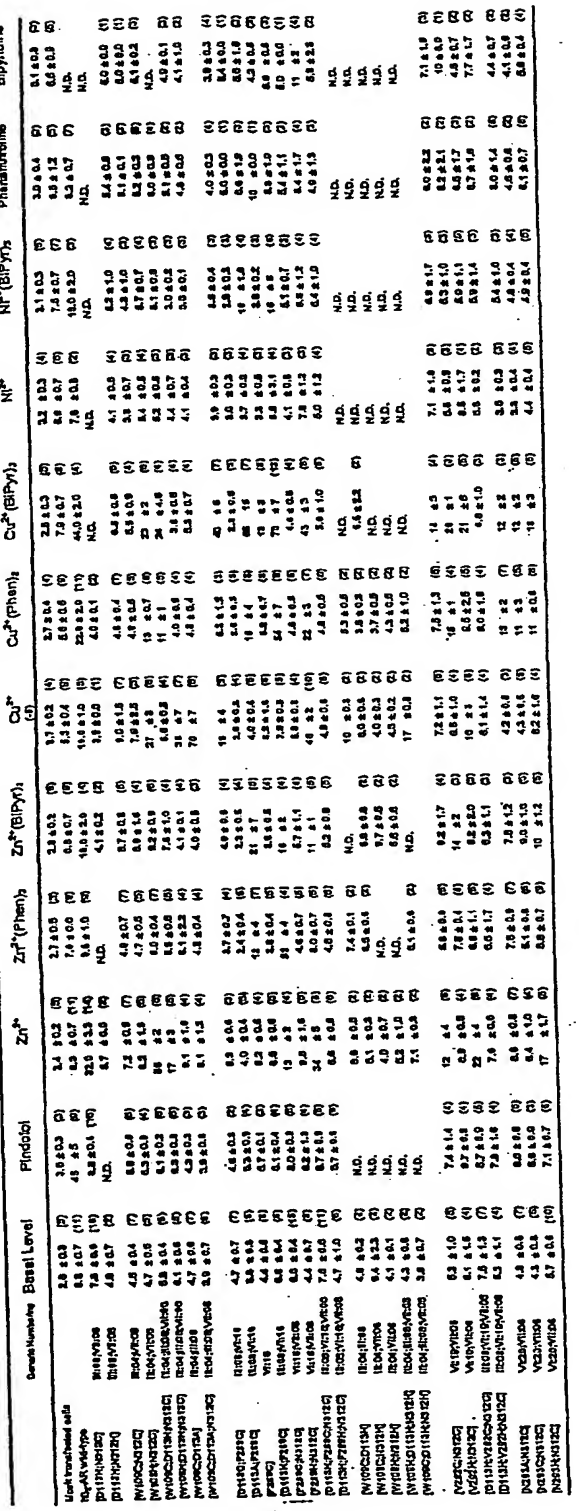
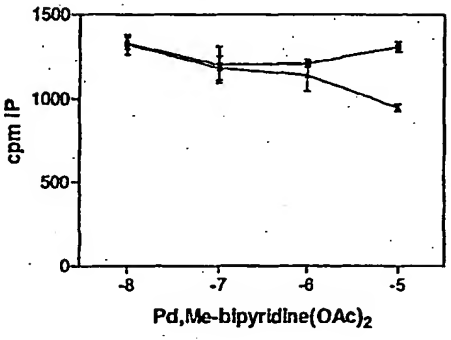
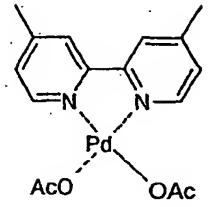
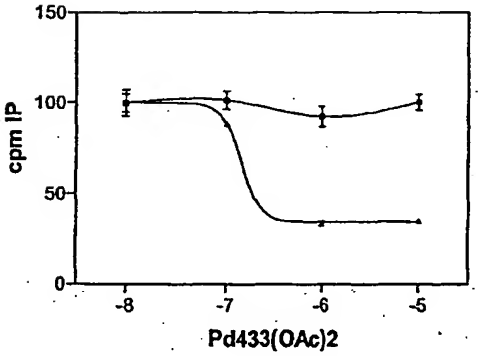


Fig. 20


1500
1000
500
0
cpm IP
-8
-7
-6
-5
Pd,Me-bipyridine(OAc)2
RO2 wt
CysVII:06


N
N
Pd
AcO
OAc


150
100
50
0
cpm IP
-8
-7
-6
-5
Pd433(OAc)2
RO2 wt
CysVII:06

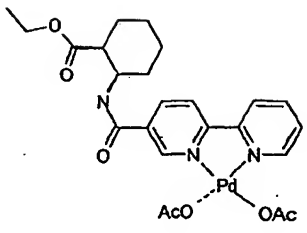

O
O
N
O
N
N
Pd
AcO
OAc

Fig. 21

INTERNATIONAL SEARCH REPORT

International Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/50 C07D207/00 C07D209/00 C07D211/00 C07D213/00
C07D215/00 C07D231/00 C07D233/00 C07D235/00 C07D241/00
C07D257/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data, BIOSIS, EMBASE, MEDLINE, WPI Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 32886 A (IGEN INT INC) 12 September 1997 (1997-09-12) page 16 -page 22; examples 15-17 page 63 -page 65 ---	1,2,4, 12, 15-27, 29,30
X	EP 0 282 893 A (BAYER AG) 21 September 1988 (1988-09-21) page 22 -page 26; examples 1-7 --- -/--	1,5,11, 13,16-25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 October 2002	11 11 2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Per Renström

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PCT/DK 02/00455

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 06260 A (CALIFORNIA INSTITUTE OF TECHNOLOGY (US)) 25 January 2001 (2001-01-25) The bipyridines at pages 31-45 and figures 2, 6, 14, 24, 32, and 50 , libraries with metal-ion chelating nitrogen ligands used in a combinatorial drug discovery method. page 25, line 23 - line 25 abstract	1,2,4, 12, 15-27, 29,30
X	WO 98 05961 A (ISIS PHARMACEUTICALS INC (US)) 12 February 1998 (1998-02-12) libraries with metal-ion chelating nitrogen ligands used in a combinatorial drug discovery method. page 1, line 17 - line 20; figures 4,10; examples	1-3,7, 21-33
X	WO 99 10016 A (RESOLUTION PHARM INC) 4 March 1999 (1999-03-04) abstract page 1 -page 4 page 30 -page 33; example 18 libraries with metal-ion chelating nitrogen ligands used in a combinatorial drug discovery method,	1,9, 16-33
X	SZURDOKI F ET AL: "A combinatorial approach to discover new chelators for optical metal ion sensing." ANALYTICAL CHEMISTRY, vol. 72, no. 21, 1 November 2000 (2000-11-01), pages 5250-5257, XP002902763 page 5254; figure 4 page 5256; figures 6,7	3,8,10, 21-23, 25-27
X	WANG F ET AL: "A Convenient Set of Bidentate Pyridine Ligands for Combinatorial Synthesis" TETRAHEDRON LETTERS, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 40, no. 26, 25 June 1999 (1999-06-25), pages 4779-4782, XP004168642 ISSN: 0040-4039 page 4780, scheme III page 4781; table 1	1,2,4, 12,15-25

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ELLING CHRISTIAN E ET AL: "Conversion of agonist site to metal-ion chelator site in the beta2-adrenergic receptor" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 96, no. 22, 26 October 1999 (1999-10-26), pages 12322-12327, XP002174721 ISSN: 0027-8424 abstract; figures 3,4 ---	1,2,5, 7-9, 11-13, 16,21-40
X	ELLING CHRISTIAN E ET AL: "Connectivity and orientation of the seven helical bundle in the tachykinin NK-1 receptor probed by zinc site engineering" EMBO JOURNAL, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 15, no. 22, 1996, pages 6213-6219, XP002174723 ISSN: 0261-4189 the whole document ---	34-40
X	ELLING CHRISTIAN E ET AL: "Disulfide bridge engineering in the tachykinin NK1 receptor" BIOCHEMISTRY, AMERICAN CHEMICAL SOCIETY. EASTON, PA, US, vol. 39, no. 4, 1 February 2000 (2000-02-01), pages 667-675, XP002174724 ISSN: 0006-2960 page 673, column 2, paragraph 2 -page 674, column 1, paragraph 1 ---	34-40
X	NORREGAARD LENE ET AL: "Delineation of an endogenous zinc-binding site in the human dopamine transporter" EMBO JOURNAL, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 17, no. 15, 3 August 1998 (1998-08-03), pages 4266-4273, XP002174722 ISSN: 0261-4189 the whole document ---	34-40
P,X	WO 01 50127 A (7TM PHARMA (DK)) 12 July 2001 (2001-07-12) the whole document -----	1-40

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PCT/DK 02/00455

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-40
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-40

Present claims 1-5, 11 and 16-40 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

The initial phase of the structural part of the search focused on what was considered to be the first apparently searchable part of the invention, namely the invention according to claim 12. However, the search revealed a very large number of documents relevant to the issue of novelty. While being structural searchable, claim 12 yields so many documents relevant to the issue of novelty upon searching, that it is impossible to determine which parts of claim 12 may be said to define the subject-matter for which protection might legitimately be sought (Article 6 PCT). For these reasons, a meaningful search over the whole breadth of claim 12 is actually impossible. The same situation was found to prevail also for each of the claims 6-10 and 13-15.

Present claims 34-40 relate to an extremely large number of possible combinations of amino acid sequences defining binding sites. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the combinations claimed, namely the combinations listed in claim 37. Regardless of this however, such lists of the combinations are not searchable. Thus, the lists of combinations of amino acid sequences defining binding sites in the present claims 34-40 have not been searched.

Present claims 34-40 do not reflect the inventive concept of the drug discovery process described in the application in a clear and concise way (Article 6 PCT). Furthermore, claims 34-40 are not clear and concise since they relate to "any of items 1-40". Claims should not refer to subsequent claims.

Present claims 3, 8 and 10 can not be subordinated to claim 1 (see the definition av X-F-Y in claim 1).

What has been performed is basically a broader search not focused on specific structures but instead on, for example, combinations of key terms like combinatorial, libraries, drug discovery, target validation, dentate and chelate, etc.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/DK 02/00455

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		WO 0150127 A2	12-07-2001
		EP 1242824 A2	25-09-2002
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